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UNIVERSITY OF ALBERTA

Elucidation of the *cyt-2-1* mutation of *Neurospora crassa*

BY

Mariola Elzbieta Drygas

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

FALL, 1990

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To my parents.

Abstract

The nuclear *cyt-2-1* mutation of *Neurospora crassa* is characterized by slow growth, female infertility and gross deficiencies of spectrally detectable cytochromes *c* and aa_3 (cytochrome *c* oxidase). Previous workers attributed the deficiency of cytochrome aa_3 to the lack of processing of cytochrome *c* oxidase subunit I, which is thought to impair the assembly of the cytochrome *c* oxidase complex.

Investigation of the cytochrome *c* deficiency in *cyt-2-1* showed that the translatable message for cytochrome *c* is produced in *cyt-2-1* cells and that apocytochrome *c* is synthesized in the mutant but is rapidly degraded in the cytosol. This suggested that transport of apocytochrome *c* into mitochondria may be impaired in *cyt-2-1*. Analysis of the *cyt-2-1* mitochondria by others revealed that they are devoid of cytochrome *c* heme lyase (CCHL) activity. CCHL catalyzes attachment of a functional heme group to the apocytochrome *c*, which is required for its transport into mitochondria.

The *cyt-2* gene was cloned by *Neurospora* transformation using a sib-selection procedure. The 2.1 kb HindIII fragment that rescues the *cyt-2-1* mutant phenotype was sequenced and an open reading frame with similarity to the amino acid sequence of the yeast CCHL was identified. The extent of the coding sequence and the position of the introns were confirmed by sequencing the *cyt-2* cDNA clone. Sequencing of the *cyt-2-1* mutant allele revealed a two base pair (CT) deletion which causes a frame shift and generates an early stop codon in the coding sequence. This results in a

truncated *cyt-2* gene product which is evidently enzymatically inactive. The results of both the analysis of the cytochrome *c* deficiency and cloning of the $cyt\text{-}2^{+}$ gene led to the conclusion that the *cyt-2-1* mutation affects the gene for cytochrome *c* heme lyase. This suggests that the cytochrome aa_3 deficiency in *cyt-2-1* is due to a secondary effect of the mutation.

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Abbreviations

Amp^R	ampicillin resistance
ATPase	adenosine triphosphatase
bp	base pair
bisacrylamide	N,N'-methylenebisacrylamide
Bn^R	benomyl resistance
bromphenol blue	3',3'',5',5''-tetrabromophenol-sulfonphthalein
CCHL	cytochrome *c* heme lyase
cDNA	complementary DNA
cpm	counts per minute
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddATP	2',3'-dideoxyadenosine 5'-triphosphate
ddCTP	2',3'-dideoxycytidine 5'-triphosphate
ddGTP	2',3'-dideoxyguanosine 5'-triphosphate
ddTTP	2',3'-dideoxythymidine 5'-triphosphate
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
dH_2O	distilled water
dITP	2'-deoxyinosine 5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dTTP	2'-deoxythymidine 5'-triphosphate
E. coli	*Escherichia coli*

EDTA	ethylenediaminetetraacetic acid, disodium salt
FGSC	Fungal Genetics Stock Center
IPTG	isopropyl-β-D-thiogalactoside
Kan^R	kanamycin resistance
kb	kilobases
kDa	kiloDaltons
mCi	milliCurie
MOPS	3-(*N*-morpholino)propanesulfonic acid
mRNA	messenger RNA
MW	molecular weight
NADH	nicotinamide adenine dinucleotide, reduced form
NADP	nicotinamide adenine dinucleotide phosphate
N. crassa	*Neurospora crassa*
NTP	nucleotide triphosphates
PEG	polyethylene glycol
rDNA	ribosomal deoxyribonucleic acid
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNP	ribonucleoprotein
rpm	revolutions per minute
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
Rubisco	ribulose 1,5-bisphosphate carboxylase

S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Tet^R	tetracycline resistance
Tris	tris (hydroxymethyl) aminomethane
Triton X-100	octylphenoxypolyethoxyethanol
tRNA	transfer ribonucleic acid
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Introduction

Mitochondria are essential organelles used by the cell for respiration and as the location of important metabolic pathways. Within this organelle are the enzymes of the citric acid cycle, enzymes required for the biosynthesis of some amino acids, nucleotides, phospholipids, heme and other metabolites. The electron transport chain that drives energy production in the process of oxidative phosphorylation is contained in the mitochondrial inner membrane (Tzagoloff, 1982).

Mitochondria are not synthesized *de novo*. Instead, they grow and are maintained by incorporating newly synthesized material into pre-existing organelles (Luck, 1963; 1965). The number of mitochondria within a cell is maintained by division and fusion events. In the steady state there is a constant synthesis and turnover of mitochondrial proteins (Posakony *et al.*, 1977). Formation of mitochondria and their functions depend on the products of both nuclear and mitochondrial genes. In order to balance production of proteins encoded in two different genomes, expression of their genes has to be coordinately regulated. This requires regulatory factors as well as a communication system between the three cellular compartments: the nucleus, the cytosol, and the mitochondrion, that are involved in control of mitochondrial protein synthesis. In addition, both mitochondrial and nuclear encoded products have to be transported to their correct location in the mitochondria where they function as either structural or enzymatic components. Considering the above, it is not surprising that mitochondrial biogenesis is a complex process which presents a cell with many problems not encountered in the production of cytosolic proteins. Mutants affecting various aspects of mitochondrial biogenesis and functions have been isolated in many organisms, most notably yeast and *Neurospora crassa*.

My project focuses on nuclear-mitochondrial gene interactions in the synthesis of mitochondrial proteins involved in respiratory functions. The subject of my study was the nuclear mutation *cyt-2-1* of *N.crassa*, which affects two components in the electron transport chain of the respiratory system, cytochromes *c* and aa_3 (cytochrome *c* oxidase). Both cytochromes are absent in the *cyt-2-1* mitochondria, as judged by spectrophotometric analysis. Deficiency of cytochrome aa_3 has been attributed to the lack of processing of the mitochondrially synthesized subunit I, which apparently impairs the assembly of the cytochrome *c* oxidase complex (Bertrand and Werner, 1979). The reason for the cytochrome *c* deficiency in the *cyt-2-1* mitochondria was not known, but could be an alteration in any of the steps of the cytochrome *c* biosynthesis. As a product of a nuclear gene, cytochrome *c* is synthesized in the cytosol and is imported into mitochondria. The fact that a single nuclear mutation affects two proteins, products of a mitochondrial and a nuclear gene, suggested that the *cyt-2* gene encodes a regulatory function involved in interactions between nuclear and mitochondrial genomes. Elucidation of the *cyt-2-1* mutation through cloning and sequencing of the *cyt-2* gene and the mutant allele, as well as investigation of the nature of the cytochrome *c* deficiency in the *cyt-2-1* mitochondria constituted my project. In the introduction to the study of the *cyt-2-1* mutation, I would like to present an overview of the current state of knowledge on the aspects of mitochondrial biogenesis relevant to this project.

Mitochondrial genome

The mitochondrion has its own genome (Luck and Reich, 1964) and all the enzymatic machinery necessary for transcribing and translating the genetic information. Mitochondrial genomes range in size from 16.5 kb in humans (Anderson *et al.*, 1981) to over 250 kb in plants (Palmer and Shields, 1984). Fungal mitochondrial genomes are intermediate in size and vary from 17 kb to 100 kb (Clark-Walker and Sriprakash, 1982; Sederoff, 1984). Despite the size variation, mitochondrial DNAs code for a similar complement of proteins. These usually include cytochrome *b*, cytochrome *c* oxidase subunits I, II and III, ATPase subunits VI and VIII, several subunits of the NADH dehydrogenase complex, as well as mitochondrial tRNAs, rRNAs and a ribosomal protein (Breitenberger and RajBhandary, 1985). The exact complement of the proteins encoded by mitochondrial genomes does vary among different organisms. For example, all vertebrate mitochondrial DNAs so far sequenced contain seven genes encoding subunits of the respiratory chain NADH dehydrogenase (Chomyn *et al.*, 1985; 1986). Homologous genes exist in the mitochondrial genomes of *N. crassa* and *Aspergillus nidulans* (Breitenberger and RajBhandary, 1985; de Vries *et al.*, 1986; Brown *et al.*, 1985). However, only a subset of these genes has been found in the mitochondrial DNA of trypanosomatides (Benne, 1985; Simpson, 1986), *Chlamydomonas* (Gray and Boer, 1988) and plants (Stern *et al.*, 1986). The mitochondrial DNAs of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* seem to lack NADH dehydrogenase genes altogether (Lang *et al.*, 1983; Grivell, 1987). In addition to the common set of genes, a number of open reading frames (ORFs) are unique to the mitochondrial genomes of fungi and plants. Some of these sequences, present within the introns of some yeast mitochondrial genes, have been found to encode proteins involved in mRNA processing or intron transposition (Kotylak *et al.*, 1985; Macreadie *et al.*, 1985; Jacquier and Dujon, 1985; Colleaux *et al.*, 1986; Lazowska *et al.*, 1989).

Dependence of mitochondria on nuclear gene products

Despite the fact that mitochondria have their own genome, their biogenesis and function are largely dependent on the expression of nuclear genes. This is most clearly illustrated in yeast *rho*^{o} mutants whose mitochondria have lost all of their DNA. Because of the absence of the mitochondrial genes that specify respiratory functions, *rho*^{o} strains can only grow anaerobically. Despite their missing genomes, mitochondria in *rho*^{o} cells develop into morphologically normal structures (Dujon, 1981) and, except for the few respiratory and ATPase proteins encoded in the mitochondrial genome, have most of the enzymatic activities present in wild-type organelles (Schatz *et al.*, 1972).

Numerous nuclear genes required for mitochondrial functions have been identified in yeast through the isolation of *petite* (*pet*) mutants, which were initially found by Ephrussi (1953). Cells that carry *pet* mutations have respiratory-incompetent mitochondria and are unable to grow on nonfermentable carbon sources. When grown on low glucose solid media they form small colonies and are therefore referred to as nuclear petites, in contrast to cytoplasmic petites which arise from large deletions in the mitochondrial genome. *PET* genes may code for products that have a direct role in mitochondrial respiration and oxidative phosphorylation or they may affect these functions indirectly. For example genes coding for the components of the mitochondrial genetic system, such as RNA polymerase (Greenleaf *et al.*, 1986; Mueller *et al.*, 1987), ribosomal proteins (Myers *et al.*, 1987) or aminoacyl-tRNA synthetase (Koerner *et al.*, 1987) qualify as *PET* genes since a defect in mitochondrial protein synthesis leads to a respiratory-deficient phenotype. Also, genes whose products act during transport of proteins into mitochondria, such as the matrix-localized protease that removes presequences from imported precursor polypeptides (Böhni *et al.*, 1983; Yaffe *et al.*, 1985), fall into this category.

In most cases nuclear genes required for the expression of specific mitochondrial genes have been found to act at the post-transcriptional steps such as RNA processing, translation, protein maturation and subunit assembly (Fox, 1986; Tzagoloff and Myers, 1986; Attardi and Schatz, 1988). Examples of genes involved in processing of particular mitochondrial transcripts in yeast are *MRS3*, *MRS1* and *CBP2* which are required for the removal of the first, third and fifth intron, respectively, from cytochrome *b* pre-mRNA (Schmidt *et al.*, 1987; Kreike *et al.*, 1986; McGraw and Tzagoloff, 1983). The product of another gene, *CBP1*, is necessary for the formation of the correct 5' end of the cytochrome *b* transcript (Dieckmann *et al.*, 1982). Mutants in the *CBP1* gene that lack this processing function produce unstable transcripts subject to nucleolytic degradation (Dieckmann *et al.*, 1984).

There are number of nuclear *pet* mutants that affect translation of specific mitochondrial transcripts (Fox, 1986; Attardi and Schatz, 1988). Three genes are known to be required for the translation of the *COX3* mRNA, which encodes subunit III of cytochrome *c* oxidase (Fox, 1986). The product of the *PET494* gene activates translation of the *COX3* transcript by directly interacting with its 5'-leader sequence (Mueller and Fox, 1984; Constanzo and Fox, 1986). In addition to *PET494*, at least two other genes, *PET54* and *PET122* are required for translation of *COX3* (Constanzo *et al.*, 1986; Constanzo and Fox, 1988). Interestingly, the *PET54* gene product is also necessary for efficient excision of one of the introns from the *COX1* gene, which encodes subunit I of cytochrome *c* oxidase (Valencik *et al.*, 1989). Multiple nuclear gene products are also involved in translation of the transcripts for cytochrome *b* and subunit II of cytochrome *c* oxidase (COXII) (Fox, 1986; Attardi and Schatz, 1988). The product of *PET111* has been shown to interact specifically with the 5'-untranslated leader of the *COX2* transcript. Mutations in the *PET111* gene can be suppressed by a fusion of the amino-terminal portion of another mitochondrial gene, for example *COX1* or ATPase 9, to *COX2*, which releases the *COX2* transcript from its control by the *PET111* product (Poutre and Fox, 1987).

An example of a nuclear gene that exerts its effect at the post-translational level of the synthesis of mitochondrially encoded proteins is the recently isolated *SCO1* gene (Schulze and Rödel, 1989). The product of the *SCO1* gene is necessary for the accumulation of subunit I (COXI) and subunit II (COXII) of cytochrome *c* oxidase (Schulze and Rödel, 1988; 1989). Unlike *pet111*, mutations in the *SCO1* gene cannot be suppressed by rearrangements in the mitochondrial DNA. This leads to the conclusion that the *SCO1* product acts at the post-translational step in COXII synthesis (Schulze and Rödel, 1988; 1989). It has been suggested that the SCO1 protein, which is found tightly associated with mitochondrial membranes, may participate in formation of a cytochrome *c* oxidase pre-complex (Schulze and Rödel, 1989). Another nuclear gene that encodes a product required for the post-translational processing of a mitochondrially encoded protein is *COR1*. The *COR1* gene encodes a 44 kDa protein that processes apocytochrome *b* to mature cytochrome *b*, most likely through the addition of the heme group (Tzagoloff *et al.*, 1986).

Mutations in nuclear genes that affect expression of mitochondrial genes have been also described for *N. crassa*. (Bertrand *et al.*, 1977; Pittenger and West, 1979), although the number of these mutants is much smaller than in yeast. Several nuclear genes that control synthesis of the mitochondrial gene products in *N. crassa* have been recently identified. Three genes, *cyt-4*, *cyt-18* and *cyt-19* define trans-acting components involved in splicing group I introns in the mitochondrial genes of *N. crassa*. Initially identified as required for splicing the intron from the gene encoding the mitochondrial large rRNA (Mannella *et al.*, 1979; Bertrand *et al.*, 1982), they were subsequently found to act in splicing of a number of group I introns from mitochondrial mRNAs (Collins and Lambowitz, 1985; Lambowitz *et al.*, 1985; Dobinson *et al.*, 1989). The product of the *cyt-4* gene seems to be necessary for correct synthesis of the 3' end of the precursor of the large rRNA, since a mutation in this gene results in a 3' extension of the transcript. Thus, it is thought that the lack of splicing of the intron in the 25S rRNA gene may be a secondary effect due to improper folding of the precursor RNA (Garriga *et al.*, 1984). Interestingly, *cyt-18* was found to encode mitochondrial tyrosyl-tRNA synthetase, which seems to exert a direct effect on splicing group I introns in *Neurospora* mitochondria (Akins and Lambowitz, 1987). Biochemical and immunological analysis of the *cyt-18* product confirmed earlier genetic evidence that the splicing activity of the tyrosyl-tRNA synthetase is separate from its synthetase activity (Majumder *et al.*, 1989). The role of the *N. crassa* *cyt-19* gene product is still unknown but it has been postulated that it may promote binding of the splicing activity, associated with the tyrosyl-tRNA synthetase, to mitochondrial RNPs (Garriga and Lambowitz, 1986; Akins and

Lambowitz, 1987).

Another gene that affects synthesis of all mitochondrial translation products in *N. crassa* is *cyt-21*. This gene was found to encode mitochondrial ribosomal protein S-24 (Kuiper *et al.*, 1988). No nuclear genes that specifically affect translation of polypeptides synthesized in mitochondria have been identified in *Neurospora* so far.

The mitochondrial genome affects expression of nuclear genes

In addition to the large number of nuclear genes that exert control over mitochondrial gene products, several findings suggest that mitochondria can also influence nuclear gene expression. In yeast, respiratory deficiency induced by drugs that specifically interfere with mitochondrial functions, affects the levels of cytochrome *c* expression in the cell nucleus (Siemens *et al.*, 1980). In *Neurospora*, chloramphenicol inhibition of mitochondrial translation in wild-type cells leads to the increased synthesis of a number of nuclear encoded mitochondrial components, including mitochondrial ribosomal proteins, cytochrome *c*, alternative oxidase and possibly mitochondrial RNA polymerase (Lambowitz and Slayman, 1971; Barath and Kuntzel, 1972a, 1972b; Kuiper *et al.*, 1988). Such activation of unlinked nuclear genes encoding mitochondrial components could result from inhibition of synthesis of a mitochondrially synthesized repressor protein (Barath and Kuntzel, 1972b), or from metabolic changes resulting from impaired mitochondrial functions (Lambowitz and Zannoni, 1978).

The status of the mitochondrial genome itself can affect expression of nuclear genes (Parikh *et al.*, 1987). Using cDNA subtraction procedures, it was shown that the abundance of some transcripts of the nuclear genome varied several fold in yeast cells with identical nuclear but different mitochondrial genotypes. Among these differently regulated transcripts were RNAs, that were derived from the so-called nontranscribed spacer (NTS) region of the nuclear rDNA repeat (Parikh *et al.*, 1989). These results suggest that the nucleus monitors the mitochondrial genome in some direct fashion which may involve a mitochondrially encoded protein that would act as a messenger (Parikh *et al.*, 1987).

Recently, it has been demonstrated for the first time that a mitochondrially encoded peptide has a function outside of the organelle. Maternally transmitted factor (MTF) is a component of the minor histocompatibility antigen (Mta) expressed on the surface of lymphocytes and fibroblasts in mice (Fischer Lindahl *et al.*, 1983; 1986). Cytoplasmic inheritance of the MTF implied that it was likely to be a mitochondrial gene product (Fischer Lindahl and Hausmann, 1983; Fischer Lindahl, 1985). Comparison of the sequences of the mitochondrial DNAs from four different MTF types that exist in mice allowed the identification of the gene in the mitochondrial genome that encodes MTF (Loveland *et al.*, 1990). A hydrophobic peptide derived from the amino terminal end of an NADH dehydrogenase subunit, encoded by the ND1 gene, was found to be responsible for the maternally inherited antigenicity (Loveland *et al.*, 1990). The mechanisms by which MTF arises is not known at present. One hypothesis suggests that such a peptide could be generated during the process of autophagic recycling of the cell's constituents, including mitochondria (Loveland *et al.*, 1990).

Communication between mitochondrion and nucleus via physiological signals

The fact that genetic information is distributed between two spatially separated compartments implies the existence of mechanisms for ensuring coordinate expression of the genes located in the two genomes. The process of communication between mitochondrion and nucleus has been studied in the greatest detail in *Saccharomyces cerevisiae*. In most cases, the communication has been found to occur at the level of proteins or metabolites that can diffuse or be transported across the membranes (Forsburg and Guarente, 1989). Synthesis of these regulatory elements is, in turn, regulated in response to physiological signals, such as oxygen levels and carbon source. Both these signals are involved in respiration: oxygen directly, as an electron acceptor in the oxidative phosphorylation pathway, and carbon source indirectly, through catabolite repression of genes encoding mitochondrial proteins (Forsburg and Guarente, 1989).

The response of a cell to the presence or absence of oxygen in most cases is mediated by the cofactor heme. Heme, which forms the prosthetic groups of cytochromes, is synthesized in mitochondria, from where it emanates to influence transcription of nuclear genes. Heme levels indirectly reflect levels of oxygen in the cell since several enzymes that act late in the heme biosynthetic pathway are oxygenases and require molecular oxygen as one of their substrates. During anaerobic growth cells are not able to synthesize heme and accumulate porphyrins instead (Mattoon *et al.*, 1979). Thus, lack of heme in wild-type cells indicates lack of oxygen.

Heme/Oxygen

Heme has long been implicated in the regulation of many hemoprotein genes in *S. cerevisiae*, such as *CTT1*, encoding catalase T (Richter *et al.*, 1980; Hamilton *et al.*, 1982) and *CYC1*, encoding iso-1-cytochrome *c*, which is a major form of cytochrome *c* in yeast (Guarente and Mason, 1983). Yeast *hem1* mutant strains which are deficient in the synthesis of the heme precursor, δ-aminolevulinic acid, show a 100-fold reduction in the expression of a *CYC1-lacZ* reporter gene, as measured by both the levels of β-galactosidase activity and mRNA (Guarente and Mason, 1983). If heme is added to cultures of *hem1* mutant strains or to cells growing anaerobically, transcription of the *CYC1* gene is induced (Guarente and Mason, 1983). Heme regulation of the *CYC1* gene is mediated by HAP1 (heme activator protein) which binds to an upstream activation site (UAS1), one of the two independent UAS elements of the *CYC1* gene (Guarente *et al.*, 1984; Pfeifer *et al.*, 1987). HAP1 also binds in a heme dependent manner to the UAS of the *CYC7* gene, encoding iso-2-cytochrome *c* which is a minor form of the cytochrome *c* in yeast (Prezant *et al.*, 1987), the *CTT1* gene (Winkler *et al.*, 1988) and other genes involved in electron transport that are induced by heme (Schneider, 1989). *CYC7* gene, which is expressed at a very low level in aerobically growing cells, is also regulated negatively by heme via the ROX1 product (Wright and Zitomer, 1984; Zitomer *et al.*, 1987). Transcription of the *ROX1* repressor gene itself was shown to be activated by heme, although it is not known whether this activation requires HAP1 (Lowry and Zitomer, 1988).

Recently, another regulatory protein, HAP2, has been reported to mediate heme activation of several genes encoding respiratory proteins, namely *COX4*, *COX5a* and *COX6* which encode subunit IV (Schneider, 1989), an isoform of subunit V (Trueblood *et al.*, 1988) and subunit VI (Trawick *et al.*, 1989) of cytochrome *c* oxidase, respectively. At present it is not known whether HAP2 acts directly on these genes as a transcriptional activator, or if it acts indirectly through some other factors (Trawick *et al.*, 1989).

Oxygen induction mediated by heme has been demonstrated in yeast for *CYC1* and *CYC7* (iso-1-- and iso-2-cytochrome *c*, respectively) (Pfeifer, 1988; Lowry and Zitomer, 1988), as well as for the *COX5* genes (Va and Vb subunits of cytochrome *c* oxidase) (Hodge *et al.*, 1989). The products of these genes are directly involved in electron transport and oxidative phosphorylation. In addition, oxygen was also shown to induce expression of the *ROX1* and *REO1* genes, which encode repressors of several genes expressed in the absence of oxygen such as *CYC7* and *COX5b* (Lowry and Zitomer, 1988). Thus, the latter genes are negatively regulated by oxygen and heme. Both *CYC7* and *COX5b* encode minor forms of two respiratory proteins, cytochrome *c* and subunit V of cytochrome *c* oxidase, respectively (Sherman and Stewart, 1971; Cumsky *et al.*, 1985). Under aerobic conditions *CYC7* and *COX5b* are negatively regulated by ROX1 and REO1, and are expressed at low levels (Lowry and Zitomer, 1988; Trueblood *et al.*, 1988). The majority of the cytochrome *c* and subunit V of cytochrome *c* oxidase in the presence of oxygen is formed by their isoforms, which are encoded by the *CYC1* and *COX5a* genes, respectively (Sherman and Stewart, 1971; Cumsky *et al.*, 1985). Under anaerobic conditions induction of the *CYC1* and *COX5a* genes does not take place because of lack of heme, whereas *CYC7* and *COX5b* genes are derepressed and their products are synthesized (Lowry and Zitomer, 1988; Trueblood *et al.*, 1988).

To account for the inverse regulation by oxygen of the two isoforms of cytochrome *c* and cytochrome *c* oxidase subunit V, it was suggested that the minor isoforms may be more efficient than the major forms under weakly anaerobic conditions (Trueblood *et al.*, 1988; Forsburg and Guarente, 1989). For example, subunit Vb of cytochrome *c* oxidase could have a higher affinity for oxygen at low concentrations than subunit Va (Trueblood *et al.*, 1988). Consistent with this hypothesis, iso-2-apocytochrome *c* is more stable than the iso-1 form in the absence of heme cofactor, which would be expected to be present at low concentrations in relatively anaerobic conditions (Matner and Sherman, 1982; Dumont *et al.*, 1990). This type of regulation may have evolved to accommodate the cell during periods of low oxygen levels, which occur during the transition from anaerobic to aerobic growth (Trueblood *et al.*, 1988; Forsburg and Guarente, 1989; Dumont *et al.*, 1990).

Carbon

Another signal that affects genes encoding mitochondrial proteins is carbon source. Yeast cells respond to growth in glucose by up-regulating fermentative metabolism, with the consequent production of ethanol. At the same time enzymes in numerous metabolic pathways, including those involved in mitochondrial electron transport and oxidative phosphorylation, are repressed (Entian, 1986; Gancedo and Gancedo, 1986). This process, referred to as glucose (or catabolite) repression, ensures that the cell preferentially utilizes the simple sugar glucose.

Catabolite or glucose repression of genes encoding mitochondrial proteins has been evident for some time (Ephrussi and Slonimski, 1950; Polakis *et al.*, 1965; Ibrahim *et al.*, 1973; Ciriacy, 1977). Several genes, such as *CYC1*, *CYC7*, *CTT1* and *COX5a*, which encode different hemoproteins are known to be transcriptionally regulated by carbon source (Zitomer *et al.*, 1979; Guarente *et al.*, 1984; Laz *et al.*, 1984; Prezant *et al.*, 1987; Zitomer *et al.*, 1987; Winkler *et al.*, 1988; Trueblood *et al.*, 1988). Catabolite repression of enzymes early in the heme biosynthetic pathway has also been reported (Mahler and Lin, 1978; Mattoon *et al.*, 1979; Labbe-Bois *et al.*, 1983), which suggests that the heme and carbon source signals may intersect.

Release from catabolite repression of cytochrome genes proceeds via a transcriptional activation system that includes the products of the regulatory genes *HAP2*, *HAP3* and *HAP4* (Forsburg and Guarente, 1989). The products of these genes form a complex that binds to the second regulatory site in the promoter region of the *CYC1* gene, UAS2 (Guarente *et al.*, 1984). They are also required for activation of other nuclear cytochrome genes (Schneider, 1989; Trueblood *et al.*, 1988), as well as genes whose products are involved in heme synthesis (Keng and Guarente, 1987) under derepressed conditions.

In addition to the HAP2/3/4 activation complex, other gene products, namely SNF1 and SSN6, have been implicated in the regulation of glucose repression in yeast (Wright and Poyton, 1990). At present it is not clear whether these products act in connection with or separately from the HAP2/3/4 complex. It has been suggested that the *SNF1* gene product which is a protein kinase (Celenza and Carlson, 1986) may phosphorylate other regulatory proteins, such as HAP2, HAP3, HAP4 or SSN6 and thus regulate transcription of the target genes indirectly (Wright and Poyton, 1990).

As can be seen, communication between mitochondria and the nucleus is a very complex process that involves many regulatory factors which act in response to major physiological signals, such as oxygen levels and carbon source. In yeast, some of these factors have been identified, but their role in regulating expression of genes encoding mitochondrial proteins is not yet fully understood. Virtually nothing is known about the regulation of genes encoding mitochondrial components in response to physiological signals in *N. crassa*. One could assume however, that the coordinate expression of nuclear and mitochondrial genes in *N. crassa* would also proceed through a network of regulatory factors in response to physiological stimuli. Since *Neurospora* is an obligate aerobe and cannot grow in the absence of oxygen, coordinate regulation of the synthesis of mitochondrial components should be simpler than in yeast. For example, *Neurospora* contains only one form of cytochrome *c*, as well as one form of subunit V of cytochrome *c* oxidase, and thus the process of inverse regulation of different isoforms in response to the changing growth conditions, as described earlier for yeast, does not occur. On the other hand, because *N. crassa* absolutely requires oxygen for growth, the mutations affecting mitochondrial respiratory functions are either lethal or lead to the reduction, rather than a complete loss of mitochondrial functions (Bertrand and Pittenger, 1972). Consequently, isolation and studies of the mutants affecting respiratory functions in *Neurospora* are more difficult which makes progress in this area much slower than in yeast.

Mitochondrial protein import

Another important aspect of mitochondrial biogenesis is transport of proteins into the organelle. More than 90% of mitochondrial proteins are encoded in the nucleus and are synthesized on free ribosomes in the cytosol. After being released into a cytosolic pool as soluble polypeptides, they are taken up by mitochondria post-translationally (Schatz and Mason, 1974; Schatz and Butow, 1983; Schatz, 1987). The question of how these polypeptides are targeted specifically to the mitochondria and how each species is delivered selectively to one of the four internal compartments has been of interest for a long time. Due to the tremendous progress that has been made during the last few years, the process of mitochondrial protein transport is generally understood, though many questions still remain unanswered.

Most of the polypeptides that are imported into mitochondria are synthesized as precursors with N-terminal peptide extensions which are proteolytically removed during or following import (Hay *et al.*, 1984; Harmey and Neupert, 1985). The N-terminal prepiece carries targeting information which is necessary to direct imported proteins into their correct intramitochondrial location: the matrix, the inner membrane, the intermembrane space or the outer membrane (Schatz, 1987). The prepieces of proteins destined for mitochondria do not share extensive sequence homology, but they do have several similar characteristics. These include: a high content of positively charged basic amino acids (particularly arginine), the absence or near absence of negatively charged acidic amino acid residues, and a high content of hydroxylated amino acids (particularly serine) (Hurt and van Loon, 1986). Although exceptions exist (Zimmermann *et al.*, 1979b; Teintze *et al.*, 1982; van Loon *et al.*, 1983; Nishikimi *et al.*, 1986), the general trend is that the deeper into the mitochondria a protein must be imported, the more likely it is to be synthesized as a precursor of higher molecular weight. Consistent with this rule, none of the known outer membrane proteins are synthesized as higher molecular weight precursors (Nicholson and Neupert, 1988). However, even though these proteins do not have removable prepieces, specific targeting information is contained within the polypeptide (Hartl *et al.*, 1989). For example, the yeast 70-kDa outer membrane protein, which is synthesized without a cleavable presequence, contains information for targeting and sorting in its amino terminal end (Riezman *et al.*, 1983). In contrast, the ADP/ATP carrier, an inner membrane protein which also lacks a cleavable presequence, seems to have specific and sufficient targeting information within its carboxy-terminal domain (Hartl *et al.*, 1989).

In order for the polypeptides to be translocated across the membranes, they have to maintain unfolded structure (Eilers and Schatz, 1986; Chen and Douglas, 1987). Presumably, this open conformation of the polypeptides is required to keep their targeting signals exposed (Hartl and Neupert, 1990). It has been shown that a family of heat shock proteins, hsp70, is among cytosolic components that prevent folding of the precursors in the cytosol (Deshaies *et al.*, 1988a; Chirico *et al.*, 1988). Import of the F_1-ATPase subunit β into mitochondria requires hsp70 proteins, and is dependent on nucleotide triphosphates (Deshaies *et al.*, 1988b). Earlier, it was demonstrated that hydrolysis of NTPs is necessary for mitochondrial protein import (Pfanner and Neupert, 1986; Pfanner *et al.*, 1987). Stuart (1989) speculates that heat shock proteins bind to the precursor polypeptides in a co-translational manner to prevent their folding and aggregation. Upon delivery of the bound precursors, in a translocation-competent form, to the surface of the outer membrane the heat shock proteins would be released concomitant with NTP hydrolysis. Release of heat shock protein from its substrate has been shown to require hydrolysis of ATP (Lewis and Pelham, 1985).

Recognition of proteins destined for import into mitochondria is facilitated by receptor proteins on the outer surface of mitochondrial membranes (Zwizinski *et al.*, 1984; Pfanner and Neupert, 1990). The receptors recognize only precursors, they do not bind mature polypeptides (Harmey and Neupert, 1985). Some receptors bind only one particular protein, for example the receptor for apocytochrome *c* (Zimmermann *et al.*, 1981; Hennig *et al.*, 1983). Others are less specific and serve as binding sites for more than one protein (Harmey and Neupert, 1985; Hay *et al.*, 1984). From the receptors the precursor proteins are transferred to a component in the outer membrane called GIP (general insertion protein) (Pfanner *et al.*, 1988; Pfaller *et al.*, 1989). Except for cytochrome *c*, all precursors that have been studied to date require the presence of an active GIP to mediate their insertion into the mitochondrial membrane (Pfaller *et al.*, 1989).

Translocation of the bound precursor proteins across the inner mitochondrial membrane in most cases depends on energy in the form of a membrane potential (Schatz and Butow, 1983; Harmey and Neupert, 1985). Generally, energy is required for all proteins with an N-terminal extension and for proteins which are inserted into or translocated across the inner membrane (Nicholson and Neupert, 1988). Proteins which are imported into the intermembrane space but do not come in contact with the inner membrane, such as cytochrome *c* (Zimmermann *et al.*, 1981), and those imported into the outer membrane, such as porin (Freitag *et al.*, 1982; Mihara *et al.*, 1982), do not require an energized membrane for import.

Polypeptides of the outer membrane, such as the yeast 70-kDa protein are inserted directly into their target membrane (Riezman *et al.*, 1983; Hase *et al.*, 1984). Their insertion may be explained by a "stop-transport" model which postulates the existence of a "stop-transfer" domain in the presequences of precursor proteins that are translocated into mitochondrial membranes (Hurt and van Loon, 1986). These stretches of hydrophobic amino acids, which are potential membrane spanning domains, would prevent complete translocation of the precursors across the mitochondrial membrane. In support of this model, the amino terminal targeting domain of the yeast 70-kDa outer membrane protein has been found to contain a hydrophobic stretch of 27 amino acid residues (Hurt *et al.*, 1985). This potential "stop-transport" domain, which follows a short matrix targeting domain, is thought to be responsible for anchoring the precursor to the outer membrane and thus preventing complete transport of the 70-kDa protein into the matrix (Hurt *et al.*, 1985).

Polypeptides destined for the matrix are transported across the two mitochondrial membranes at contact sites, where the outer and inner membranes come close enough together to be simultaneously spanned by the imported polypeptide (Nicholson and Neupert, 1988). Upon entering the matrix, the N-terminal presequences of the precursor polypeptides are proteolytically cleaved by a metal-dependent processing peptidase (MPP) located in the matrix (Böhni *et al.*, 1983; Schmidt *et al.*, 1984). Cleavage activity of the MPP is stimulated by the processing-enhancing protein (PEP) which may also act directly in translocation of the polypeptides by interacting cotranslationally with their N-terminal presequences (Hawlitschek *et al.*, 1988; Hartl *et al.*, 1989).

Most inner membrane and intermembrane space proteins follow a two step translocation process referred to as "conservative sorting" (Hartl *et al.*, 1989). These proteins are first translocated across both membranes at their contact sites into the matrix. After proteolytic processing of the precursor polypeptide by the matrix protease, the intermediate precursors are then retranslocated to the outer side of the inner membrane or intermembrane space (Hartl *et al.*, 1989). Often a second proteolytic cleavage of the polypeptide also occurs, catalyzed by another processing peptidase localized at the outer surface of the inner membrane (Hartl *et al.*, 1987). The characteristics of the transport from the matrix strongly resemble those of protein export from the cytoplasm across the plasma membrane in prokaryotes (Hartl *et al.*, 1986; 1987). It is believed that this part of the transport into the inner membrane and the intermembrane space has been conserved since the endosymbiotic origin of mitochondria, and thus the process has been named "conservative sorting" (Hartl *et al.*, 1989). "Conservative sorting" has been demonstrated to be part of the mitochondrial import pathway for several inner membrane and intermembrane space proteins such as cytochrome b_2, cytochrome c_1 and others (Hartl *et al.*, 1986; Pfanner *et al.*, 1987; Hartl *et al.*, 1987).

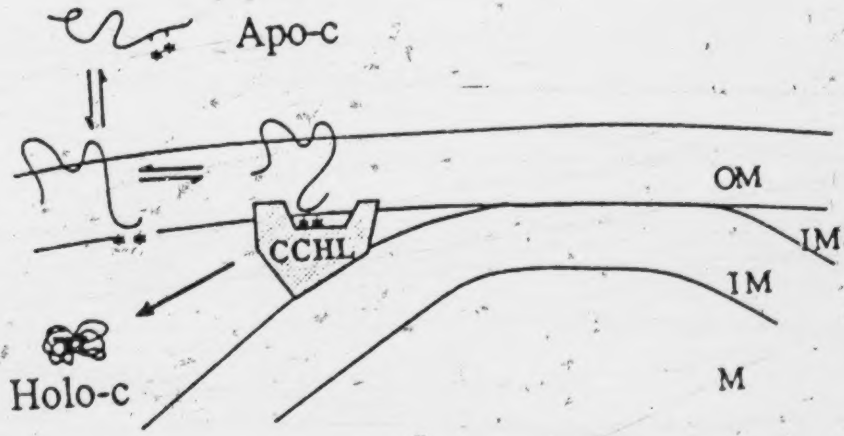
The imported polypeptides are sometimes modified further by covalent or non-covalent attachment of cofactors, such as heme or iron-sulfur, and undergo conformational changes (Hartl *et al.*, 1989). Many polypeptides are also assembled into larger complexes composed of several different subunits, for example, the cytochrome *c* oxidase or the ATPase complexes. Very little is known about the mechanisms underlying folding and assembly of mitochondrial proteins. Recently, it has been found that the nuclear encoded mitochondrial heat shock protein hsp60 is required for the assembly of several proteins imported into the mitochondrial matrix (Cheng *et al.*, 1989). The equivalent of the hsp60 has been detected in mitochondria from several sources, including those of human cell lines (Hemmingsen *et al.*, 1988; Jindal *et al.*, 1989). Structurally these proteins are related to "chaperonins" such as the *E. coli* heat-shock protein groEL and the α component of the Rubisco subunit-binding proteins. Chaperonins are a class of molecular chaperons found in chloroplasts, mitochondria and prokaryotes which assist the post-translational assembly of oligomeric protein structures (Hemmingsen *et al.*, 1988). The mechanism of their action is as yet unknown. It has been suggested that folding and assembly of the matrix, and possibly other mitochondrial proteins, does not occur spontaneously, but rather is a protein-catalyzed process (Cheng *et al.*, 1989; Ostermann *et al.*, 1989).

Cytochrome *c*

Cytochrome *c*, one of the components of the electron transport chain, is encoded by a nuclear gene and is transported into the mitochondrial intermembrane space by a distinct pathway (Fig. 1) (Nicholson *et al.*, 1987; Hartl *et al.*, 1989; Stuart *et al.*, 1990). The polypeptide is synthesized on cytoplasmic ribosomes without an N-terminal presequence (Zimmermann *et al.*, 1979a; Smith *et al.*, 1979) and is released as a soluble protein into a cytoplasmic pool (Korb and Neupert, 1978). No protease-sensitive components for binding apocytochrome *c* have been found on the surface of the mitochondria (Nicholson *et al.*, 1988). Instead, apocytochrome *c* can spontaneously insert into the lipid bilayer of the outer mitochondrial membrane (Berkhout *et al.*, 1987; Mannella *et al.*, 1987), where it is subsequently bound by a specific receptor (Hennig and Neupert, 1981; Hennig *et al.*, 1983). There is evidence that cytochrome *c* heme lyase is part of this complex, since mitochondria devoid of cytochrome *c* heme lyase activity are also deficient in binding apocytochrome *c* (Nargang *et al.*, 1988; Nicholson *et al.*, 1988). It has been suggested that in addition to its catalytic function, cytochrome *c* heme lyase may act as a receptor for the apocytochrome *c*

Figure 1. Transport of cytochrome *c* into mitochondria (Stuart *et al.*, 1990).

Apocytochrome *c* (apo-c), the precursor of holocytochrome *c* (holo-c) is synthesized on free cytosolic ribosomes. After being released into a cytosolic pool the polypeptide partially inserts into the mitochondrial outer membrane (OM) where it binds to cytochrome *c* heme lyase (CCHL) which acts as a specific receptor. Translocation across the outer membrane into the intermembrane space (IMS) is thought to be driven by the refolding of the cytochrome *c* polypeptide as a result of covalent heme attachment. Cysteine sulfhydryl residues to which the heme becomes attached are indicated by asterisks.


Apo-c
OM
CCHL
IMS
IM
Holo-c
M

(Nicholson *et al.*, 1988; Stuart *et al.*, 1990).

Unlike other imported mitochondrial proteins which have to cross the outer membrane, cytochrome *c* does not require membrane potential nor ATP for translocation (Zimmermann *et al.*, 1981; Pfanner and Neupert, 1985; Stuart *et al.*, 1990). The fact that apocytochrome *c* transport is independent of NTPs suggests that heat shock proteins may also not be needed to maintain the unfolded structure of the polypeptide (Stuart, 1989). Nicholson *et al.* (1988) suggested that a specific conformation of apocytochrome *c*, rather than the unfolded structure, is required for its targeting into mitochondria.

The covalent attachment of heme to apocytochrome *c*, crucial for its role in electron transport, also appears important for the translocation of the polypeptide across the outer mitochondrial membrane (Nicholson *et al.*, 1987). When heme attachment is inhibited by the analogue deuterohemin, the import of cytochrome *c* is blocked at the stage of high affinity receptor binding. Upon reversal of deuterohemin inhibition with protohemin, the bound apocytochrome *c* is converted to holocytochrome *c* and the protein is translocated completely across the membrane (Hennig and Neupert, 1981). It is believed that formation of the thioether bonds between the heme vinyl groups and the thiols of two cysteines of apocytochrome *c* initiates conformational changes along the polypeptide chain, which results in driving the translocation of the protein across the outer membrane (Nicholson *et al.*, 1987). It appears that the membrane insertion properties of the apocytochrome *c* molecule substitute for a receptor/GIP system and that free energy, generated during refolding of the polypeptide after heme attachment drives the protein across the outer membrane. Thus cytochrome *c* was able to escape the "conservative sorting" pathway of the mitochondrial import (Stuart *et al.*, 1990).

The object of this study

The object of this study is the *cyt-2-1* mutant of *N. crassa*, first described by Mitchell *et al.* (1953). The mutant is characterized by slow growth, female infertility and gross deficiencies of spectrally detectable cytochromes *c* and aa_3 (Fig. 2) (Mitchell *et al.*, 1953). Both, cytochrome *c* and cytochrome aa_3 (cytochrome *c* oxidase) are components of the mitochondrial electron transfer chain and thus affect respiratory functions.

Cytochrome *c* acts as an electron carrier between cytochrome *c* reductase (bc_1 complex) and cytochrome *c* oxidase (cytochrome aa_3) (Fig. 3) (Chance and Hess, 1959; Estabrook, 1961; Hackenbrock and Hammon, 1975). Cytochrome *c* oxidase is the terminal enzyme in the respiratory chain and catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen (Fig. 3) (Hackenbrock and Hammon, 1975; Kadenbach and Merle, 1981; Tzagoloff, 1982; Capaldi *et al.*, 1983). The enzyme is located in the mitochondrial inner membrane and in *Neurospora*, yeast and mammals is composed of at least 8 subunits (Werner, 1977; Power *et al.*, 1984; Kadenbach and Merle, 1981). In addition to the polypeptide subunits, the holoenzyme contains four electron acceptors, two heme groups and two copper atoms (Capaldi *et al.*, 1983; Denis, 1986). The three largest subunits: I, II and III, are encoded by the mitochondrial genome and are synthesized inside the organelle (Sebald *et al.*, 1973; Schatz and Mason, 1974). In *Neurospora* subunits I and II are synthesized with N-terminal leader sequences that are subsequently cleaved by an as yet unknown processing enzyme (Machleidt

Figure 2. Cytochrome spectra obtained from the mitochondria of wild-type (A) and *cyt-2-1* (B) *Neurospora crassa* strains.

The positions of the α-bands of cytochromes aa_3 (608 nm), *b* (560 nm) and *c* (550 nm) are indicated.

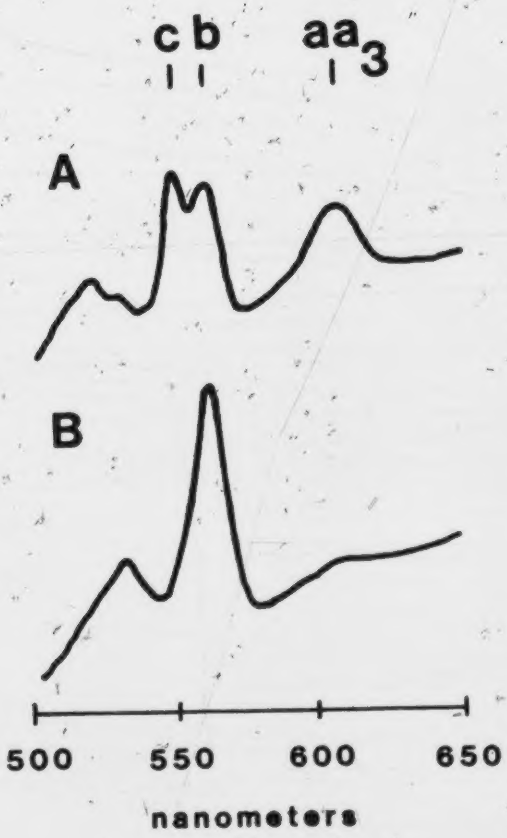
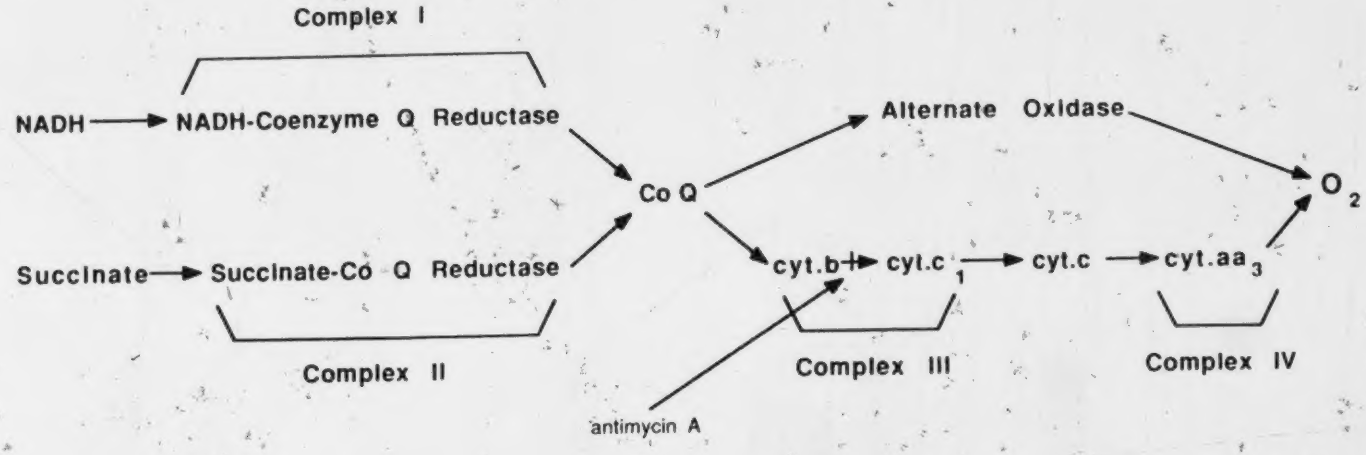

c
b
aa$_3$
A
B
500
550
600
650
nanometers

Figure 3. Electron transport chain of *Neurospora* mitochondria.

(References: Lambowitz and Slayman, 1971; Bertrand *et al.*, 1976; Tzagoloff, 1982).


Complex I
NADH
NADH-Coenzyme Q Reductase
Succinate
Succinate-Co Q Reductase
Complex II
Co Q
Alternate Oxidase
O_2
cyt.b
cyt.c_1
cyt.c
cyt.aa_3
antimycin A
Complex III
Complex IV

and Werner, 1979). The remaining subunits are encoded in the nuclear genome and imported into mitochondria post-translationally (Tzagoloff *et al.*, 1979).

It has been shown by immunological methods that the *cyt-2-1* mutant does not possess fully assembled cytochrome *c* oxidase. This deficiency has been correlated with the lack of processing of the mitochondrially synthesized subunit I of cytochrome *c* oxidase, which is present in a high molecular weight form in the mutant (Bertrand and Werner, 1979). The estimated size of the subunit I polypeptide from *cyt-2-1* cells is 45 kDa, whereas the mature form present in wild-type mitochondria has a molecular weight of 41 kDa (Bertrand and Werner, 1979). This unprocessed subunit I could affect proper assembly of cytochrome *c* oxidase in the *cyt-2-1* mutant. A similar defect in subunit I processing has also been observed in the cytochrome aa_3-deficient mitochondrial mutant [*mi-3*] (Bertrand and Werner, 1979; Werner and Bertrand, 1979). DNA sequence analysis of the *oxi-3* gene, encoding the subunit I polypeptide, in the [*mi-3*] mutant revealed a missense mutation in the coding sequence that leads to an amino acid substitution in the mature subunit (Lemire and Nargang, 1986). It has been proposed that this amino acid substitution causes a conformational change in the polypeptide altering a protease recognition and/or binding site and therefore preventing efficient processing of the subunit (Lemire and Nargang, 1986). However, this model cannot explain the cytochrome aa_3 deficiency in the *cyt-2-1* strain since the *cyt-2-1* mutation is in a nuclear gene. It also does not explain the lack of cytochrome *c*, which could not be immunoprecipitated from either mitochondria or cytosol of *cyt-2-1* mutant cells (Nargang *et al.*, 1988).

In both the *cyt-2-1* and [*mi-3*] mutants cytochrome aa_3 synthesis can be induced by chemical or genetic means that block the flow of electrons in the cytochrome bc_1 region of the electron transport chain (Fig. 3) (Bertrand and Collins, 1978). It was shown that low levels of antimycin A (0.3 μg/ml) or the presence of the nuclear *cyb-1-1* allele that affects synthesis of cytochrome *b*, restores levels of cytochrome aa_3 to normal in the [*mi-3*] mutant, and partially in the *cyt-2-1* mutant (Bertrand and Collins, 1978). Interestingly, although cytochrome *b* deficiency persists in the *cyb-1-1*, [*mi-3*] double mutants, *cyb-1-1*, *cyt-2-1* strains are not deficient in cytochrome *b* (Bertrand and Collins, 1978). Neither the presence of the *cyb-1-1* mutation, nor antimycin A alleviates the cytochrome *c* deficiency in the *cyt-2-1* mutant (Bertrand and Collins, 1978).

The fact that a single mutation affects two polypeptides, one being the product of a nuclear gene (cytochrome *c*) and the other the product of a mitochondrial gene (cytochrome *c* oxidase subunit I), suggested that the *cyt-2* gene may encode a regulatory protein required for accumulation of both cytochromes. However, since antimycin A or the *cyb-1-1* mutation can alleviate the cytochrome aa_3 deficiency, but does not restore cytochrome *c* in *cyt-2-1*, it may be that the mutation affects these cytochromes in two different ways.

Two approaches were taken in elucidating the nature of the *cyt-2-1* mutation of *N. crassa*. One approach, in which the cytochrome *c* deficiency was examined, was aimed at determining at what step cytochrome *c* biosynthesis is altered in the mutant strain. In an attempt to answer this question, cytochrome *c* transcription, translation and transport into mitochondria (the latter done by others) were examined in *cyt-2-1* cells. The second approach was to clone and sequence the *cyt-2*$^+$ gene in order to gather information about the gene product. Once the *cyt-2*$^+$ gene was identified and its coding sequence confirmed by sequencing the *cyt-2* cDNA clone, the mutant allele was also cloned and the primary lesion of the *cyt-2-1* mutation determined. The results of both studies led to the conclusion that the *cyt-2-1* mutation affects the gene for cytochrome *c* heme lyase. This conclusion suggests that the cytochrome aa_3 deficiency in *cyt-2-1* is due to a secondary effect of the mutation.

Materials and Methods

N. crassa strains and culture conditions

The strains of *N. crassa* used in this study are described in Table 1. The wild-type strain 240*A* is referred to as NCN10 in our laboratory. Mutant strains, *cyt-2-1 a* (NCN68), *cyt-2-1 pan-2 a* (NCN69), and *cyt-2-1 pan-2 inl a* (NCN241), were derived from the following crosses. A *cyt-2-1 pan-2 a* strain (IS-4), obtained from H. Bertrand (University of Regina), was crossed with the wild-type strain 74-OR23-1*A* (74*A*). One of the single ascospore progeny of this cross, *cyt-2-1 pan-2 a* (NCN44), was then crossed with a *qa-2 aro-9 pab inl A* strain (NCN40), obtained from Dr. R. Akins (St. Louis University). NCN68 and NCN69 were single ascospore isolates from this cross. Strain NCN241 is a single ascospore isolate from a subsequent cross of a *qa-2 aro-9 pab inl A* strain (NCN40) with NCN69. All three *cyt-2-1* strains may also carry either the *qa-2* or *aro-9* mutation, but not both since they do not require aromatic amino acids for growth. The IS-4 and NCN44 strains were lost during storage.

Strains used for mapping by restriction fragment length polymorphism (RFLP) were obtained from the Fungal Genetics Stock Center (FGSC, Strain numbers 4410 through 4430). These included two parental and 18 progeny strains from a cross between a laboratory strain RLM1-33*a* (Oak Ridge) and a strain Mauriceville-1c*A* (Metzenberg *et al.*, 1984, 1985).

N. crassa cultures were grown in liquid or solid Vogel's medium

Table 1. *N. crassa* strains.

Strain	Genotype	Source
240*A*	*nic-1 al-2 A*	Dr. H. Bertrand
NCN40	*qa-2 aro-9 pab inl A*	Dr. R. Akins
NCN68	*cyt-2-1 a*	see text
NCN69	*cyt-2-1 pan-2 a*	see text
NCN241	*cyt-2-1 pan-2 inl a*	see text

containing appropriate supplements (Davis and de Serres, 1970). Cultures for the generation of conidia were grown on Vogel's medium solidified with 1.5% (w/v) agar (Sigma), in 250 ml Erlenmeyer flasks and were incubated at 30°C When the mycelia had covered the medium at the bottom of the flask, they were taken out of the incubator and allowed to conidiate. Liquid Vogel's medium was inoculated with conidia (*ca*. 10^6/ml) and incubated at 25° to 30°C in a shaker-incubator until the culture reached the stage of growth required for individual experiments.

E. coli strains and culture conditions

Strains of *Escherichia coli* used are described in Table 2. Strain DH1 was used for maintenance of the pSV50 cosmid (Vollmer and Yanofsky, 1986) and the *Neurospora* cosmid library; JM83, for maintenance of bacterial plasmids pUC19 (Yanisch-Perron *et al.*, 1985) and pKGS (Kuhn *et al.*, 1986), as well as any recombinant clones derived from these plasmids; JM103, to propagate M13 clones; BB4, for amplification of the *N. crassa* cDNA library; XL1-Blue, for excision and recircularization of inserts contained within the lambda ZAP vector.

Most *E. coli* strains were grown in L-broth containing appropriate antibiotics (Maniatis *et al.*, 1982). Overnight cultures of JM103, were grown in Davis minimal medium (Davis and Mingioli, 1950) containing 10 μM thiamine, 0.5% glucose. Strains BB4 and XL1-Blue were grown overnight in TB broth (5 g NaCl, 10 g bacto-tryptone per 1 liter) supplemented with 0.2% maltose and 10 mM $MgSO_4$. The cells were centrifuged in an SS-34 rotor (Sorvall) at 3,000 rpm for 10 minutes and resuspended in 0.5 volume of 10 mM $MgSO_4$.

Table 2. Bacterial strains.

Strain	Genotype	Reference
DH1	F$^-$, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, (r_k^-, m_k^+), *supE44*, *relA1?*, λ^-	Maniatis *et.al.*, 1982
JM83	*r-*, *ara*, *Δ(lac-proAB)*, *rpsL*, *thi*, *φ80*, *lacZΔM15*, *hsdR*$^-$	Yanisch-Perron *et al.*, 1985
JM103	*Δ(lac-proAB)*, *supE*, *thi*, *strA*, *sbcB15*, *endA*, [F' *traD36*, *proAB*, *lacI*^{q}, ZΔM15]	Messing,1983; Yanisch-Perron *et al.*, 1985
BB4	*supF58*, *supE44*, *hsdR514*, (r_k^-, m_k^-), *galK2*, *galT22*, *trpR55*, *metB1*, *tonA*, λ^-, Δ(*arg-lac*), *U169* [F' *proAB*, *lacI*^{q}, ZΔM15 *Tn10*(*tet*^{R})]	Predigested λ Zap/ EcoRI - Instruction Manual, Stratagene, 1987
XL1-Blue	*endA1*, *hsdR17*, (r_k^-, m_k^+), *supE44*, *thi-1*, λ^-, *recA1*, *gyrA96*, *relA1*, (*lac*$^-$), [F' *proAB*, *lacI*^{q}, ZΔM15, *Tn10*(*tet*^{R})]	Predigested λ ZAP/ EcoRI - Instruction Manual, Stratagene, 1987

Antibiotics, for selective purposes, were added as required (Table 3). For amplification of plasmids in liquid cultures, chloramphenicol was added to a final concentration of 150 µg/ml, when the culture reached O.D.$_{600}$=0.6 (Maniatis *et al.*, 1982).

Maintenance of the strains

N. crassa strains were maintained on slants containing solidified Vogel's medium with appropriate supplements, and stored in a sealable plastic container at 4°C. *Cyt-2-1* strains of *N. crassa* were transferred to fresh slants every 3 to 4 months to ensure viability of the mutant strains.

Bacterial colonies for routine usage were maintained on L-broth plates (Lennox, 1955) stored inverted at 4°C for up to 6 months. For longer storage, bacterial stocks freshly grown to saturation in L-broth were made to 15% glycerol (Maniatis *et al.*, 1982) and stored at -20°C or -70°C.

Plasmid and cosmid DNA vectors

Plasmids pUC19 (Yanisch-Perron *et al.*, 1985), pKGS (Kuhn *et al.*, 1986) and the cosmid pSV50 (Vollmer and Yanofsky, 1986) were used as cloning vectors (Table 4). The pSV50 cosmid contains, in addition to pBR322 sequences and λ *cos* sites, a *Neurospora* β-tubulin gene that confers resistance to benomyl. Recombinant plasmids constructed during the course of the work are listed in Table 5.

Table 3. Antibiotics added to bacterial media.

Antibiotics	Stock concentration (mg/ml)	Final concentration (µg/ml)
ampicillin	10	100
streptomycin	100	100
tetracycline-HCl	2	20
chloramphenicol	34	20
kanamycin	25	50

Table 4. Plasmid and cosmid cloning vectors.

Vector	Antibiotic resistance	References
pUC19	Amp^R	Yanisch-Perron *et al.*, 1985;
pKGS	Kan^R	Kuhn *et al.*, 1986;
pSV50	Amp^R, Bn^R	Vollmer and Yanofsky, 1986;

Table 5. Recombinant plasmids.

Plasmid	Parental plasmid	Description of the cloned fragments
pSV50-7617	pSV50	an insert of *ca* 38 kb carrying the *cyt-2*$^+$ gene isolated from *N. crassa* genomic library;
pUCB2	pUC19	*cyt-2*$^+$ gene on a 9.6 kb BamHI fragment subcloned from pSV50-7617;
pMD6	pUC19	*cyt-2*$^+$ gene on a 2.1 kb HindIII fragment subcloned from pUCB2;
pMDM5	pKGS	*cyt-2-1* mutant allele on a 2.1 kb HindIII fragment;
pMDC1	pBluescript[1]	1.6 kb *cyt-2* cDNA clone with EcoRI linkers; first isolate
pMDC6	pBluescript	1.6 kb *cyt-2* cDNA clone with EcoRI linkers, second isolate;
pMDC9	pBluescript	1.4 kb *cyt-2* cDNA clone with EcoRI linkers

1. See Fig. 5 for description of the plasmid.

Plasmid and cosmid DNA isolation

Plasmid and cosmid DNAs were isolated by a cleared lysate procedure (Maniatis *et al.*, 1982) and further purified by CsCl-ethidium bromide gradient centrifugation. For large scale isolation of bacterial plasmid or cosmid DNA, 200 ml of L-broth containing an appropriate antibiotic was inoculated with 2 ml of an overnight culture of *E. coli* grown in L-broth with the same antibiotic. Cells were grown at 37°C with vigorous shaking for 3 to 4 hours. At this time chloramphenicol was added to a final concentration of 150 to 180 µg/ml in order to amplify the plasmid, and the culture was further incubated at 37°C with vigorous shaking for 15 to 20 hours. Cells were then harvested by centrifugation in a GSA rotor (Sorvall) at 4°C for 5 minutes at 4,000 rpm and resuspended in 2 ml of sucrose buffer (25% sucrose, 50 mM Tris-Cl pH 8.2). 0.5 ml of a lysozyme solution (10 mg/ml in 250 mM Tris-Cl pH 8.0) was added and the mixture incubated on ice for 10 minutes, followed by addition of 1 ml of 250 mM EDTA and further incubation on ice for 10 minutes. Cell lysis was achieved by adding 4 ml of lysis buffer (2% SDS, 50 mM Tris-Cl pH 8.0, 62.5 mM EDTA), which was mixed with the cells by gentle inversion. The mixture was left on ice for 15 to 60 minutes until the solution became very viscous. To this, 0.5 ml of a 5 M NaCl solution was added to the above mixture and mixed well by inversion. The lysate was then transferred to 50Ti tubes (Beckman) and pelleted in a 50Ti rotor (Beckman) at 4°C for 45 minutes at 45,000 rpm. The supernatant was collected and 0.98 g of cesium chloride was added for each 1 ml. The CsCl was dissolved by placing the solution on an aliquot mixer until it dissolved completely. 0.5 ml of ethidium bromide solution (10 mg/ml) was then added and the solution was left in the dark at room temperature for 15 min. The insoluble material was removed by centrifugation in an SS-34 rotor (Sorvall) at 15,000 rpm for 30 minutes at room temperature. The supernatant was transferred to Quick-Seal tubes (Beckman) and the plasmid DNA was banded by equilibrium-density centrifugation in a VTi65 rotor (Beckman) at 20°C for a minimum of 6 hours at 54,000 rpm. DNA collected from CsCl-ethidium bromide gradients was extracted 3 to 4 times with salt saturated isopropanol (NaCl in 50 mM Tris-Cl pH 8.0) to remove ethidium bromide. CsCl was removed by dialysis against TE buffer (10 mM Tris-Cl pH 7.8, 1 mM EDTA). DNA was then extracted with water saturated phenol, followed by extraction with chloroform/iso-amyl alcohol (24:1) (v/v), and precipitated twice with ethanol in the presence of 2.5 M ammonium acetate. The dried pellet was dissolved in TE buffer.

Recombinant plasmid DNAs used for *N. crassa* transformation were isolated from *E. coli* by basically the same procedure, except that DNA purification by CsCl-ethidium bromide centrifugation was replaced by two precipitations as described by Akins and Lambowitz (1985). Cells of an overnight culture (10 ml) grown in L-broth with the appropriate antibiotic were pelleted in an SS-34 rotor (Sorvall) at 4°C for 5 minutes at 5 000 rpm. After resuspending the pellet in 1 ml of sucrose buffer (25% sucrose, 50 mM Tris-Cl pH 8.2), 250 µl of lysozyme solution (10 mg/ml in 250 mM Tris-Cl pH 8.0) was added and the cells were incubated on ice for 10 minutes. To this, 0.5 ml of 250 mM EDTA was added and the mixture was incubated on ice for another 10 minutes. Cell lysis was induced by adding 2 ml of lysis buffer (2% SDS, 50 mM Tris-Cl pH 8.0, 62.5 mM EDTA), and incubation on ice was continued for 15 to 60 minutes. After this time, 1 ml of 8 M potassium acetate was added and the mixture was left on ice for 30 minutes until a large precipitate formed. The chromosomal DNA and protein precipitate was removed by centrifugation in an SS-34 rotor (Sorvall) at 4°C for 10 minutes at 12,000 rpm and the nucleic acids precipitated from the supernatant by addition of 1/3 volume of 10 M ammonium acetate (or 1/10 volume of 3 M sodium acetate) and 2 volumes of iso-propyl alcohol. Following 10 minutes incubation at room temperature, the nucleic acids were pelleted by centrifugation at 10,000 rpm for 10 minutes. The dried pellet was dissolved in 300 µl H-NET buffer (100 mM Tris-Cl pH 7.8, 150 mM NaCl, 1 mM EDTA), and 15 µl of RNase A (1 mg/ml, pretreated at 100°C for 10 minutes) was added. After incubation with RNase A at 37°C for 30 minutes, 15 µl of Protease K (10 mg/ml, predigested at 37°C for 30 minutes) was added and the incubation was continued for another 30 minutes. The solution was then extracted with phenol, followed by chloroform/iso-amyl alcohol (24/1) (v/v), and the DNA was precipitated with ethanol, as described before. The dried pellet was redissolved in 50 µl of TE buffer.

Rapid plasmid DNA isolation

A rapid miniprep procedure, which is a modification of the alkaline lysis procedure of Birnboim and Doly (1979), was applied to isolate plasmid DNA for electrophoretic analysis of various recombinant plasmids and cosmids. Cells from 5 ml of the *E. coli* overnight culture were spun at 4°C for 5 minutes at 7,000 rpm in an SS-34 rotor (Sorvall). The pellet was resuspended in 200 µl of glucose buffer (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA) and transferred to a 1.5 ml Eppendorf tube. 400 µl of a freshly prepared alkaline-SDS solution (0.2 M NaOH, 1% SDS) was added to the cells, mixed by gentle inversion and left on ice for 5 minutes. To this, 300 µl of cold 3 M sodium acetate (pH 4.8) was added and mixed well by gentle inversion. After 10 minutes incubation on ice, the precipitated proteins and non-supercoiled DNA was pelleted in a microcentrifuge at 4°C for 10 minutes. 750 µl of the supernatant was transferred to a clean Eppendorf tube and 450 µl of cold isopropyl alcohol was added. The solutions were mixed well and the tube was incubated at -20°C for 5 minutes. The precipitated material was pelleted by centrifugation in a microcentrifuge at 4°C for 5 minutes. The pellet was resuspended in 200 µl of distilled water and the nucleic acids reprecipitated with cold ethanol in the presence of 2.5 M ammonium acetate (pH 7.5). After drying the pellet, plasmid DNA and the remaining RNA were dissolved in 100 µl of distilled water. 5 to 10 µl of the solution was used for each restriction digest. 2 µl of RNase A solution (1 mg/ml) was added to each digest to degrade any remaining RNA.

Bacterial transformation

Transformation of *E. coli* was performed using a modified calcium chloride procedure as described by Mandel and Higa (1970). 25 ml of L-broth was inoculated with 250 µl of a fresh overnight culture and incubated at 37°C with vigorous shaking until early logarithmic phase was reached (1.5 to 2 hours). The culture was quickly cooled on ice and centrifuged in a sterile SS-34 tube (Sorvall) at 4°C for 1 minute at 7,000 rpm. The pellet was gently resuspended in 20 ml of 10 mM calcium chloride solution and spun down for 30 seconds at 7,000 rpm. Cells were then gently suspended in 0.5 to 1 ml of 30 mM calcium chloride and left on ice for 20 to 30 minutes. To competent cells in a small volume (100 to 200 µl), 10 to 20 µl (no more than 1/10 volume of cells) of the DNA solution was added and the mixture was incubated on ice for 30 minutes. Cells were heat shocked at 37°C for 90 seconds and diluted with L-broth 3 to 5 times depending on the volume to be plated. The mixture was incubated at 37°C for 45 to 60 minutes to allow expression of the antibiotic resistance gene before plating on a selective medium.

M13 transfection

Cells of the JM103 strain were prepared for transfection with M13 single-stranded DNA as described in the procedure for bacterial transformation. Typically, 50 to 100 µl of competent cells and 1 to 300 ng of DNA solution (in less than 1/10 volume of the cell volume) were used for each transfection. After incubation on ice, aliquots of 5 to 25 µl were plated on L-agar plates in soft agar (0.7%) containing 250 µl of lawn cells from a fresh, saturated culture of JM103 strain, 10 µl of IPTG solution (24 mg/ml) and 50 µl of X-Gal solution (24 mg/ml in N,N-dimethylformamide). Plates were incubated for 8 to 14 hours at 37°C to allow formation of the plaques and development of the color, due to hydrolysis of X-Gal.

Isolation of M13 single-stranded DNA

25 ml of L-broth was inoculated with 100 µl of a fresh JM103 overnight and a single M13 plaque. After 9 to 10 hours of growth at 37°C with vigorous shaking, the cells were centrifuged in an SS-34 rotor (Sorvall) at 4°C for 30 minutes at 18,000 rpm. The supernatant was immediately transferred to another tube containing 6.5 ml of 10% PEG-8000, 2.5 M NaCl and mixed well. The solution was left on ice for 30 minutes to overnight. Precipitated phage

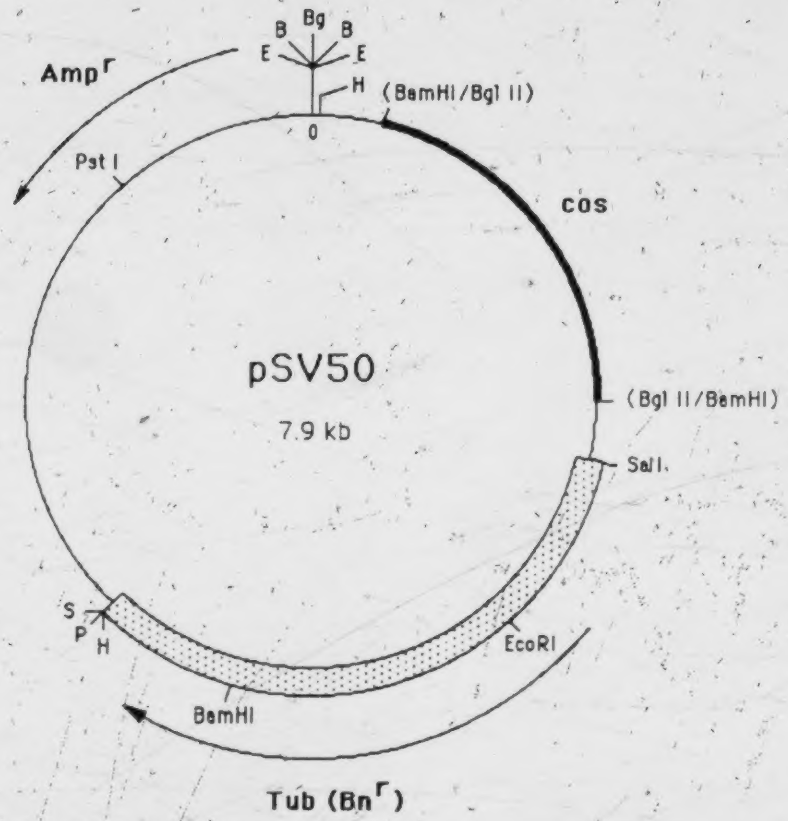
were pelleted by centrifugation in an SS-34 rotor (Sorvall) at 4°C for 20 minutes at 10,000 rpm. After removing any traces of the supernatant from the sides of the tubes with a tissue, the pellet was resuspended in 650 µl of phenol extraction buffer (300 mM NaCl, 100 mM Tris-Cl pH 7.9, 1 mM EDTA), to which 10 µl of 10% SDS and protease K (2 mg/ml) were added. The tubes were incubated at 37°C for 30 minutes. Phage proteins were removed by two phenol and one chloroform/iso-amyl alcohol (24/1) (v/v) extractions and the DNA was precipitated with ethanol. The dried DNA pellet was resuspended in 100 µl of water and 1 µl samples were run together with single-stranded DNA standards on a 0.8% agarose gel to determine the DNA concentration to be used in hybridization reactions for DNA sequencing.

N. crassa cosmid library

The *N. crassa* library, from which the *cyt-2*$^+$ gene was isolated, was constructed in cosmid pSV50 (Fig. 4) as described by Vollmer and Yanofsky (1986). Packaged recombinant cosmids were kindly provided by Drs. S. Vollmer and C. Yanofsky (Stanford University). These were used to infect *E. coli* DH1 cells which were then plated on L-agar containing ampicillin. Individual colonies were picked into the wells of microtiter dishes containing L-broth and ampicillin (100 µg/ml) and incubated overnight with gentle shaking at 37°C. To each well sterile glycerol was added to a final concentration of 15% and the dishes were stored at -70°. A library consisted of 3,024 individual clones which were preserved individually in microtiter dishes. It should be noted that this library differs from the "standard" version of the *N. crassa* pSV50 cosmid library established by Vollmer and Yanofsky

Figure 4. Map of cosmid vector pSV50.

The thin lines indicate sequences that are derived from pBR322. The thick line represents the 1.8 kb $\phi80/\lambda$ hybrid fragment containing *cos* sequence. The stippled box represents the 2.6 kb HindIII-SalI fragment of *N. crassa* DNA containing β-tubulin gene (*tub-2*) that confers benomyl resistance (Bn^R). Arrows indicate position and direction of transcription of the *E. coli* ampicillin-resistance gene (Amp^R) and the *N. crassa* β-tubulin gene. The restriction sites indicated are: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; P, PstI; S, SalI. The BglII restriction site at position 0 is the cloning site for the vector.


B
Bg
B
E
E
H
Amp^r
(BamHI/Bgl II)
0
Pst I
cos
pSV50
7.9 kb
(Bgl II/BamHI)
SalI.
S
P
H
EcoRI
BamHI
Tub (Bn^r)

(1986) in that it contains a completely separate set of cosmid isolates.

For the first round of transformation by sib selection, the library was organized into 11 sibs, 10 consisting of 288 clones each, and the 11th with 144 clones. Cells of individual clones from three consecutive microtiter plates were grown together, and the cosmid DNAs were isolated. This mixture of cosmid DNA representing each sib was used for the first round of transformation. A sib containing a clone capable of rescuing the mutant phenotype was identified after the first round of transformation. It was then subdivided into 6 sibs, each consisting of 48 clones. Again, cells of each of these sibs were grown together and the isolated cosmid DNAs were used for the second round of transformation. In subsequent rounds of transformations the number of clones was further reduced to 8 per sib (third round), and finally to 1 clone per sib (fourth round). The sib selection procedure for the *cyt-2*$^+$ gene is represented diagramatically in Fig. 8 of the Results section.

Neurospora transformation

Transformation of *N. crassa* spheroplasts was performed using the procedure of Schweizer *et al.* (1981) with the modifications described by Akins and Lambowitz (1985). Fresh *cyt-2-1* conidia (less than 2 weeks old) were harvested and 1-2 x 10^9 conidia were germinated 150 ml of in half strength Vogel's medium at 25°C with gentle shaking (200 rpm). Germination of the conidia was periodically checked under a microscope and incubation was continued until at least 80% of germination was reached (about 9 hours). At this point conidia were harvested by centrifugation in an SS-34 rotor (Sorvall) at room temperature for 10 minutes at 3,000 rpm. They were then washed 3 times with 30 ml of 1M sorbitol and the final pellet was suspended in 10 ml of 1 M sorbitol. The conidia concentration was adjusted to 2 x 10^8/ml and the suspension was transferred to a sterile 250 ml Erlenmeyer flask. Novozyme^{TM} 234 (Novo Laboratories Inc.) was added to a final concentration of 15 mg/ml and the solution was incubated in a shaker-incubator with gentle agitation (100 rpm) at 30°C for 2 hours. The resulting spheroplasts were spun down in a clinical centrifuge at the lowest setting for 12 minutes and washed twice with 10 ml of 1M sorbitol and once with 10 ml of SMC buffer (1 M sorbitol, 10 mM MOPS pH 6.3, 50 mM calcium chloride). The supernatant after each centrifugation was removed by gentle suction. The pellet was resuspended in 20 ml of SMC buffer and a sample of spheroplasts counted in a hemocytometer. The spheroplasts were then pelleted by centrifugation in a clinical centrifuge and resuspended in SMC buffer to a final concentration of 2.5 x 10^8/ml. For each 1 ml of spheroplast suspension 12.5 µl of dimethylsulfoxide and 275 µl of sterile PMC solution (40% (w/v) PEG-4000, 10 mM MOPS pH 6.3, 50 mM calcium chloride) were added and gently mixed. Aliquots of 0.25 to 1 ml spheroplasts were frozen in liquid nitrogen and stored at -70°C. Viability of the spheroplasts was tested by thawing an aliquot of the spheroplasts and plating a series of dilutions in top agar (see Appendix) onto Vogel's-sorbose plates (see Appendix). Once thawed, the spheroplasts were never refrozen for subsequent use.

Samples of the spheroplasts for transformations were thawed on ice and mixed with plasmid DNA solution that had been incubated previously with 5 µl of heparin (5 mg/ml). Transformations were carried out at a ratio of about 1 µg of DNA per 10^6 viable spheroplasts, except for the first two rounds of transformations in which 6 µg of DNA per 10^6 spheroplasts were added. In each subsequent round of transformation the amounts of DNA and spheroplasts were reduced by about half. The volume of the DNA samples was adjusted with water to equal 60% of the volume of the spheroplasts. The transformation mixture was incubated on ice for 30 minutes, after which time PMC (10 times the volume of the spheroplasts) was added, and the mixture was left at room temperature for 20 minutes. The transformation mixture was plated in top agar (see Appendix) onto benomyl containing Vogel's sorbose plates (see Appendix). For best results 1-2 x 10^5 spheroplasts in 10 ml of top agar were plated onto each plate. Plates were incubated at 30°C. Fast growing colonies were visible after 48 hours in the first round of sib selection and as early as 12 hours in the last round.

All operations were performed under sterile conditions except for the addition of the Novozyme powder.

Isolation of *N. crassa* DNA and construction of the *cyt-2-1* partial library

N. crassa DNA was isolated from the *cyt-2-1* strain (NCN69) using the procedure of Schechtman (1986). After harvesting, half of the mycelial pad from a 500 ml culture was immediately frozen in liquid nitrogen and used for DNA isolation. The remaining portion was used for analysis of mitochondrial cytochromes to monitor for possible contamination of the culture.

To construct a partial library from the mutant strain, total *cyt-2-1* DNA was digested with HindIII and electrophoresed on a 0.8% agarose gel. The fraction containing fragments from 1.8 to 2.3 kb in size was cut out of the gel and the DNA electroeluted. The recovered DNA was extracted once with phenol, once with chloroform/isoamyl alcohol (24/1) (v/v), precipitated with ethanol, and dried. The DNA was resuspended in water and ligated into the positive selection vector pKGS (Kuhn *et al.*, 1986), that had also been digested with HindIII. Plasmid pKGS carries a kanamycin resistance gene as well as an altered gene for EcoRI endonuclease under the control of the lacUV5 promoter. In the absence of EcoRI methylase the plasmid is lethal, unless a piece of foreign DNA is introduced into one of the cloning sites within the EcoRI endonuclease gene. Selection of insert-containing colonies was on L-agar plates containing kanamycin (50 µg/ml) for selection of the pKGS plasmid and IPTG (1 mM) for induction of the lacUV5 promoter.

Isolation of *N. crassa* total RNA

Total RNA was extracted by the procedure of Reinert *et al.* (1981) with minor modifications as described by Nargang *et al.* (1988). All glassware used for RNA isolation was baked at 180°C for a minimum of 3 hours to inactivate RNases. Whenever possible, solutions were treated with 0.1% diethylpyrocarbonate (DEPC) for at least 12 hours and autoclaved. Mycelium from a 14-hour wild-type culture and a 36-hour *cyt-2-1* mutant culture was harvested by filtration and immediately frozen in liquid nitrogen. Frozen mycelium was ground in a mortar to a powder with acid washed, heat sterilized Ottawa sand (1.5 g sand per 1 g of wet mycelium). For each 1 g of wet mycelium 5 ml of buffer (4% SDS, 100 mM sodium acetate, 1 mM EDTA pH 5.0) was added and grinding was continued until a smooth paste was formed. The suspension was quickly transferred to heat sterilized Corex tubes and an equal volume of phenol/chloroform/iso-amyl alcohol (25/24/1) (v/v/v) was added. Solutions were mixed for 15 to 30 minutes at room temperature and centrifuged in an SS-34 rotor (Sorvall) for 15 minutes at 10,000 rpm. The aqueous phase was removed to a clean tube and the phenol phase remaining in the tube was reextracted with 10 ml of the grinding buffer. After centrifugation the second aqueous phase was combined with the first in a clean Corex tube and predigested protease K was added to a final concentration of 0.1 mg/ml. Following 1 hour incubation with gentle mixing at room temperature, the solution was extracted twice with phenol/chloroform/iso-amyl alcohol (25/24/1) (v/v/v). The final aqueous phase was mixed with 2 volumes of ethanol, left at -20°C overnight, and then centrifuged in an SS-34 rotor (Sorvall) at 4°C for 15 minutes at 10,000 rpm. The pellet was dissolved in 5 to 10 ml of sterile water and mixed with 3 volumes of cold 4 M sodium acetate, 10 mM EDTA pH 6.0. After 10 minutes incubation on ice, precipitated material was pelleted by centrifugation for 30 minutes at 12,000 rpm. The pellet was redissolved in sterile water and, after removing 5 µl to determine RNA concentration, the RNA was reprecipitated with ethanol in the presence of 0.3 M sodium acetate pH 5.0, and stored at -70°C until further use.

Oligo-dT chromatography

Poly(A) RNA was separated from nonpolyadenylated RNA species by chromatography on oligo(dT)-cellulose (Edmonds *et al.*, 1971; Aviv and Leder, 1972), essentially as described by Maniatis *et al.* (1982). 0.2 mg of oligo(dT)-cellulose (Pharmacia LKB Biotechnology Inc., type 7) was soaked in sterile, distilled water for 10 minutes and packed in a disposable, 10 ml pipet. To such a prepared column equilibrated with loading buffer (10 mM Tris-Cl pH 7.5, 0.4 M NaCl, 0.1 mM EDTA, 0.5% SDS), 5 to 10 mg of total RNA dissolved in loading buffer was applied. After washing the column with 5 to 10 column-volumes of the same buffer, followed by 4 column-volumes of the same buffer but containing 0.1 M NaCl, poly(A) RNA was eluted with 2 to 3 ml of elution buffer (10 mM Tris-Cl pH 7.5, 0.1 mM EDTA, 0.05% SDS). Sodium acetate was added to a final concentration of 0.3 M and the RNA was precipitated with 2.2 volumes of cold ethanol. After an overnight incubation at -20°C the sample was centrifuged in an SS-34 rotor (Sorvall) at 10,000 rpm for 15 to 30 minutes at 4°C. The poly(A) RNA pellet was resuspended in distilled water and reprecipitated. Poly(A) RNA was stored at -70°C at a concentration of 1 µg/µl.

RNA electrophoresis and Northern transfer

Electrophoretic separation of RNA and transfer of RNA from the gel to the membrane was performed as described by Fourney *et al.* (1988). A 5 µl RNA sample (up to 25 µg) in water mixed with 25 µl of electrophoresis sample buffer (0.75 ml deionized formamide, 0.15 ml 10x MOPS, 0.24 ml formaldehyde, 0.1 ml deionized RNase-free water, 0.1 ml glycerol, 0.08 ml of 10% (w/v) bromphenol blue) was heat denatured at 65°C for 15 minutes and then loaded on a denaturing agarose gel. The gel was prepared by dissolving 1.0 to 1.3 g agarose in 10 ml of 10x MOPS/EDTA buffer (0.2 M 3-[N-morpholino]propanesulphonic acid, 50 mM sodium acetate, 10 mM EDTA, pH 7.0) and 87 ml of DEPC-treated, autoclaved water. After the agarose solution cooled to 50°C, 5.1 ml of 37% formaldehyde was added and the mixture was poured into a gel tray. RNA samples were loaded into the wells 1 hour later and the gel electrophoresed at

25 V at room temperature in recirculating MOPS/EDTA buffer (20 mM 3-[N-morpholino]propanesulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0), until the dye migrated about 9 cm into the gel (12 to 14 hours). A 0.24 to 9.5 kb RNA ladder (BRL) was used as a molecular weight standard. After the run was completed, the gel was transferred to Biotrans nylon membrane by capillary action, using a sponge placed in a reservoir containing 10x SSC solution (1.5 M NaCl, 150 mM sodium citrate). Following overnight transfer, the blot was baked in an oven at 80°C for 1 hour and hybridized to specific probes. A piece of the membrane containing the RNA ladder was stained with methylene blue as described by Monroy (1988) and then aligned with an autoradiograph of the remaining part of the blot.

Northern and Southern hybridizations

Northern, Southern and colony hybridizations were done as described in the protocols for Biotrans nylon membrane (ICN Biomedicals, Inc.). Hybridizations were carried out at 42°C in solutions containing 5x Denhardt's (see Appendix), 5x SSC, 50 mM sodium phosphate pH 6.5, 0.1% SDS, 250 µg/ml of nonhomologous DNA and 50% deionized formamide. The hybridization was allowed to proceed for 16 to 24 hours in the case of Northerns and Southerns, and 4 to 6 hours for colony hybridizations. After hybridization was completed, membranes were washed 4 times in 2x SSC, 0.1% SDS at room temperature for 5 minutes, and twice in 0.1x SSC, 0.1% SDS at 50°C for 15 to 30 minutes. Following the wash, membranes were subjected to autoradiography.

To remove the probe for subsequent rehybridization experiments, the membrane was incubated in 50% formamide, 10 mM sodium phosphate pH 6.5 at

65°C for 1 hour and washed in 2x SSC, 0.1% (w/v) SDS at room temperature for 15 minutes with vigorous agitation.

Radiolabeling of DNA

Plasmid or restriction fragment probes for hybridizations were uniformly labeled using random oligonucleotide primers, as described by Feinberg and Vogelstein (1983; 1984). 10 to 50 ng of DNA template in a 15 μl volume was boiled for 2 minutes and quickly cooled on ice. To this, 10 μl of 5x oligo labeling buffer (see Appendix), 5 μl of bovine serum albumin (4 mg/ml), 5 μl of [α-^{32}P]dCTP (3000 Ci/mmol) and 13 μl distilled water was added. The reaction was started by addition of 2 units of Klenow fragment of DNA polymerase I and was allowed to proceed at room temperature overnight. The reaction was stopped by addition of 200 μl of stop buffer (20 mM NaCl, 20 mM Tris-Cl pH 7.5, 2 mM EDTA, 0.25% SDS, 1 μM dCTP). Labeled DNA was purified from unincorporated nucleotides by spun-column chromatography, as described by Maniatis *et al.* (1982), using Sephadex G-50. To determine the incorporation of the radioactivity into labeled DNA, 1 μl samples were spotted onto two DE81 ion exchange Whatman filter papers. One of the papers was washed with 50 ml of 0.3 M ammonium formate solution, which removes remaining unincorporated nucleotides. The number of counts incorporated into labeled DNA was determined from this filter. Both filters were counted in a Beckman LS7500 liquid scintillation counter after addition of 5 ml of scintillation fluid (Aqueous Counting Scintillant, Amersham). The difference between the number of counts on the unwashed and washed filters reflected the efficiency with which the unincorporated nucleotides were removed by spun-column chromatography.

Strand specific probes were synthesized using appropriate clones of either M13 mp18 or mp19 single stranded DNA as the template, hybridized to a specific primer (P.L. Biochemicals, Inc., Catalog No. 1544). 0.5 µg of template was mixed with 10 ng of primer in the presence of 1 µl of 10x polymerase buffer (70 mM Tris-Cl pH 7.5, 70 mM $MgCl_2$, 500 mM NaCl) and distilled water to make a final volume of 8 µl. Annealing was carried out by incubation of the mixture at 65°C for 10 min, followed by slow cooling to room temperature. To begin the polymerization reaction, 1 µl of a 0.5 mM solution of each dATP, dTTP and dGTP; 2 µl (20 µCi) of [α-^{32}P]dCTP (3000 Ci/mmol) 1 µl 100 mM dithiothreitol, and 1 µl (1 unit/µl) of the Klenow fragment of DNA polymerase I were added. After incubation for 1 hour at room temperature, an additional 1 unit of Klenow was added and incubation continued for another hour. The reaction was terminated by the addition of 1 µl 250 mM EDTA pH 8.0 and 2 volumes of 95% ethanol. The DNA was pelleted by centrifugation, washed with 70% ethanol, dried, and resuspended in 50 µl of TE buffer. The single stranded M13 probes were used in hybridization reactions without denaturing.

Primer for primer extension reactions was labeled at its 5' end essentially as described by Geliebter (1987). A 1 µl sample (0.1 µg) of oligonucleotide, 20mer (Regional DNA Synthesis Laboratory, Calgary) was radiolabeled in a reaction mix containing 2.4 µl of 5x kinase buffer (250 mM Tris-Cl pH 7.4, 50 mM $MgCl_2$, 25 mM dithiothreitol, 0.5 mM spermidine), 0.6 µl of T4 polynucleotide kinase (9,100 units/ml) (Pharmacia) and 8 µl of [γ-^{32}P]dATP (3000 Ci/mmol) (New England Nuclear, Inc.). The reaction was incubated for 30 minutes at 37°C and stopped by heating to 65°C for 5 minutes. 8 µl of sterile water was added to make a final concentration of oligonucleotide 5 ng/µl. The incorporation of counts into the oligonucleotide was determined as described in the previous section.

DNA sequencing

DNA sequences were determined by the dideoxy chain termination method (Sanger *et al.*, 1977). The M13 phage vectors mp18 and mp19 (Yanisch-Perron *et al.*, 1985) were used for the generation of single stranded template DNA which was then annealed with appropriate M13 primers (New England BioLabs, Inc. and Regional DNA Synthesis Laboratory, Calgary). The annealing mixtures contained 0.5 to 1 μg of a single-stranded template DNA, 5 ng of a primer and 1.5 μl of 10x polymerase buffer (70 mM Tris-Cl pH 7.5, 70 mM $MgCl_2$, 500 mM NaCl) in a total volume of 15 μl. The sample was heated in boiling water for 2 minutes or at 70°C for 10 minutes and then left at room temperature for 15 to 30 minutes to allow annealing of the primer to the template. The hybridization mixture was then transferred to a Nunc Microwell Plate (60 wells), and 1 μl of 0.1 M dithiothreitol, 1 μl of radioisotope, and 1 μl of Klenow fragment (1 unit) were added. Either [α-^{32}P]dATP (600 Ci/mmol) (NEN) or [α-^{35}S]dATP (1000 Ci/mmol) (Amersham) was used as a radioactive label. Aliquots of 3 μl were dispensed into four adjacent wells and the elongation reaction was initiated by the addition of an equal volume of the A, C, G or T mixes (see Appendix). After mixing gently by the action of a Pipetman, the reactions were incubated in an air incubator at 42°-51°C (higher temperatures were used when problems with secondary structures were encountered). After 10 to 20 minutes, 1 μl of dNTP chase solution (see Appendix) was added to each well and the incubation continued for another 10 to 20 minutes. The reactions were stopped by adding 14 µl of dye-formamide mix (see Appendix) to each well. The samples were transferred to Eppendorf tubes, denatured by placing in hot water (70-90°C) for 3 minutes and loaded onto 6% (w/v) polyacrylamide denaturing gels (see Appendix). Short gels (40 cm x 20 cm x 0.3 mm) were electrophoresed at 25 Watts (constant power setting) and long gels (80 cm x 20 cm x 0.3 mm) at 50 Watts, in 0.1 M Tris-borate pH 8.3, 2 mM EDTA. In cases where regions of compression were encountered, reaction mixes containing dITP instead of dGTP were used to sequence the region (Mills and Kramer, 1979; Sanger *et al.*, 1982).

When [α-^{35}S]dATP was used, gels were dried at 80°C in a Slab Dryer (Bio-Rad) Model 483, and autoradiography performed at room temperature for 24 to 36 hours. ^{32}P gels were not dried, and autoradiography was performed at -20°C for 16 to 20 hours. XAR-5 X-ray film (Kodak) was used for autoradiography and was developed according to the manufacturer's instructions.

Primer extension

Primer extension reactions were performed essentially as in the procedure for hybridization and primer extension by Williams and Mason (1985). Annealing of the primer (complementary to the 5' end of the *cyt-2* coding sequence) to the poly(A) RNA template was carried as follows. *N. crassa* poly(A) RNA was precipitated with ethanol to remove any traces of SDS. After centrifugation, the pellet was dissolved in sterile, DEPC-treated water and an equal volume of the 2x annealing buffer (500 mM KCl, 20 mM Tris-Cl pH 8.3) was added to give a final RNA concentration of 1 µg/µl. 10 µl of the RNA solution (10 µg) was combined with 1 µl (5 ng) of an end labeled specific primer (page 57) and heated at 80°C for 3 minutes. Annealing was carried out for 4 hours at 50°C. Hybridization temperature was calculated using the following formula: 4 x (GC) + 2 x (AT) - 5 (Geliebter, 1987), and further adjusted in the course of experiment depending on the results obtained. The reaction was carried out in an Eppendorf tube and contained: 5 µl of 1 M Tris-Cl pH 8.3, 10 µl of 100 mM dithiothreitol, 6 µl of 100 mM $MgCl_2$, 2.5 µl of actinomycin D (1 mg/ml; Sigma), 5 µl of 10 mM dATP, dCTP, dTTP, 10 µl of 10 mM dGTP, and 30 µl of DEPC-treated, sterile water. To this, 11 µl of the annealed primer-RNA template and 10 units of AMV reverse transcriptase (Pharmacia) were added. After 1 hour incubation at 45°C, 4 µl of RNase A (10 mg/ml) were added and incubation continued for 20 minutes at 37°C. Primer extension products were extracted once with phenol/chloroform/iso-amyl alcohol (25/24/1) (v/v/v) and once with chloroform/iso-amyl alcohol (24/1) (v/v). They were then ethanol precipitated in the presence of 2.5 M ammonium acetate, dried and resuspended in 3 µl of sterile water. After adding 6 µl of dye-formamide mix (see Appendix), the sample was denatured by heating at 90-95°C for 3 minutes, and then electrophoresed on a 6% sequencing gel (see Appendix) together with a sequencing reaction initiated from the same primer. Gels were dried and autoradiography performed as described in the protocol for RNA sequencing.

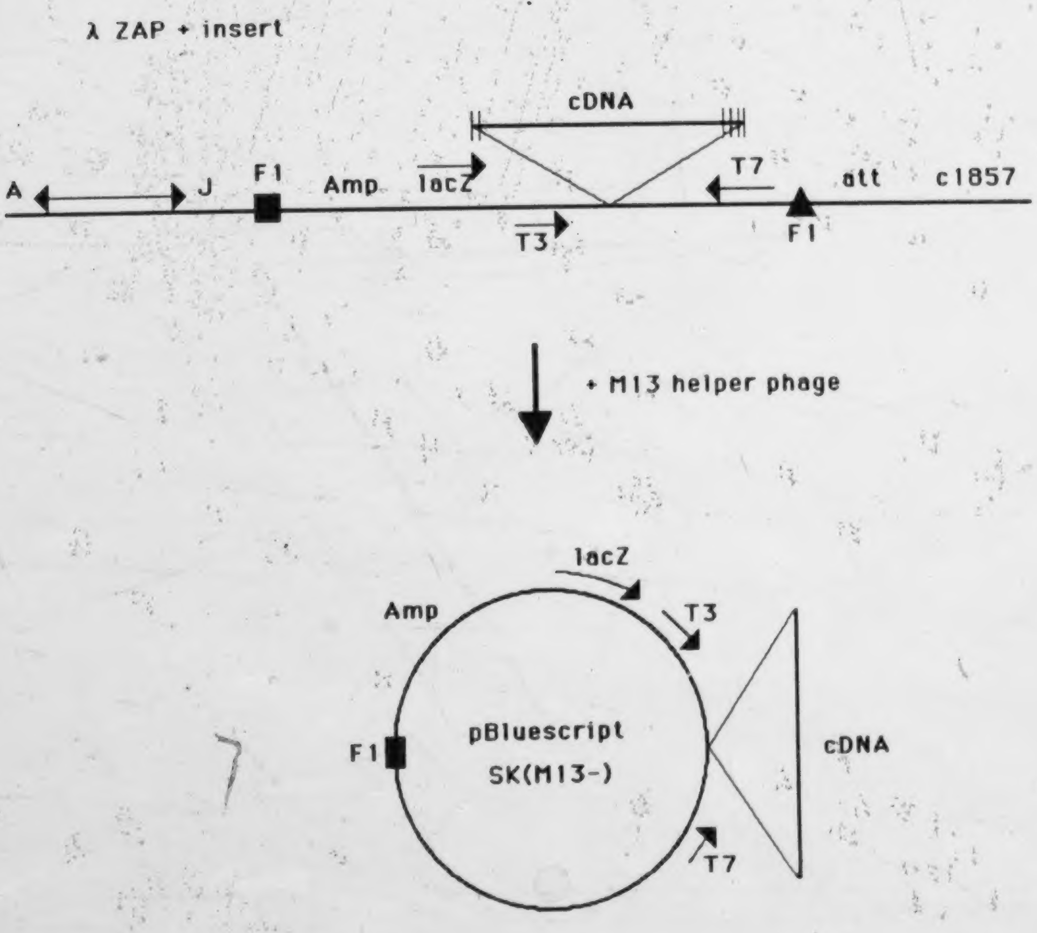
Isolation of the *cyt-2* cDNA clone from the λ ZAP library.

A *N.crassa* cDNA library constructed in the λ ZAP/EcoRI vector (Stratagene) was generously provided by Dr. Matthew Sachs (Stanford, California). *Cyt-2* cDNA clones were isolated from the library according to the instructions from the Stratagene Manual, as follows. 50 µl of a phage stock (6.4 x 10^{10} pfu/ml) diluted 10^{-4} in SM buffer (see Appendix) was added to 0.5 ml of BB4 host cells suspended in 10 mM $MgSO_4$ to $O.D._{600}=0.5$. After 15 minutes of incubation at 37°C, the mixture was plated in top agarose (see Appendix) onto two large (500 cm^2) LB plates that were one day old (see Appendix). Plates were incubated at 37°C until plaques just appeared (6 to 8 hours) and then left at 4°C for at least 2 hours, before transferring the plaques onto Biotrans (ICN Biomedicals, Inc.) nylon membrane. Filters were then processed following the protocol for Colony and Plaque Lifts (page 65) and hybridized to a 2.1 kb HindIII probe containing the *cyt-2* gene. Plaques that gave a positive signal from the hybridization reaction were further purified and rescreened until single well isolated plaques could be identified. These plaques were picked with a sterile Pasteur pipette and suspended in 0.5 ml of SM buffer. Each phage stock was titered and subjected to an *in vivo* excision process designed to remove the cloned insert from the λ ZAP phage vector and to generate a recombinant phagemid with the cDNA now carried by the vector pBluescript SK (M13$^-$). Within the recombinant λ ZAP genome, the pBluescript and cDNA insert sequences are flanked by sequences that can serve as the sites of initiation and termination for bacteriophage f1 replication (Fig. 5). If λ ZAP phage DNA is exposed to f1 derived proteins by simultaneously infecting a strain of *E. coli* with both the λ ZAP recombinant phage and a f1 (or M13) helper bacteriophage, the helper phage products recognize the f1 phage initiation and termination signals within this lambda vector and replicate one strand of the DNA between these sequences. The resulting single stranded DNA molecule, which includes sequences of the phagemid and the insert, is circularized by the gene II product of the helper phage. The circularized DNA is packaged by f1 (or M13) coat proteins and released from *E. coli*. *E. coli* cells

Figure 5. Automatic excision of cDNA inserts from λ ZAP vector.

Excision of cDNA inserts occurs after simultaneous infection of *E. coli* cells with λ ZAP recombinant phage and the M13 (or f1) helper phage. M13 derived proteins recognize F1 initiation (■) and termination (▲) sequences for DNA replication placed in the λ ZAP vector and duplicate the DNA region between the sequences. The resulting single stranded DNA molecule is circularized by gene II product of the helper phage and forms pBluescript SK(M13$^-$) plasmid which contains the inserted sequence.

Abbreviations: A-J, lambda structural genes; att, lambda attachment site; T3/T7, RNA polymerase promoter.


λ ZAP + insert
cDNA
A
J
F1
Amp
lacZ
T7
att
c1857
T3
F1
+ M13 helper phage
lacZ
Amp
T3
F1
pBluescript
SK(M13-)
cDNA
T7

infected by packaged phagemids convert the single stranded DNA to double stranded DNA and maintain it as a plasmid.

40 µl of a single λ ZAP phage stock (>10^5 pfu/ml) and 2 µl of a helper phage M13KO7 (2 x 10^9 pfu/ml) were added to 40 µl of the XL1-Blue host cells (O.D.$_{600}$=1.0) in a test tube (it is important to use a large enough tube for the excision reaction to allow good aeration of the cells). After 15 minutes of incubation at 37°C, 1 ml of 2x TY medium (see Appendix) was added and the mixture incubated at 37°C with vigorous shaking for 6 hours. Samples were then heated at 70°C for 20 minutes to kill the *E. coli* cells, transferred to Eppendorf tubes and microcentrifuged for 5 minutes. The supernatant, containing single stranded pBluescript plasmids packaged in M13 phage particles, was collected in a fresh tube. To infect cells and induce plasmid formation, 100 µl of the collected supernatant was combined with 100 µl of the BB4 cells (tet^R) (O.D.$_{600}$=1.0). After 15 minutes incubation at 37°C, 1 to 100 µl aliquots were plated onto LB plates containing ampicillin and tetracycline. Colonies appearing on the plate should contain the pBluescript plasmid with the cloned DNA insert. Because the negative control for the excision and rescue process (XL1-Blue cells and M13KO7 helper phage alone, no recombinant λ ZAP phage) gave colonies on LB-amp-tet plates, further tests were done on the colonies that were expected to contain the *cyt-2* cDNA insert on a pBluescript plasmid. Six colonies from each plate were patched onto another LB-amp-tet plates, grown for 4 to 5 hours and transferred onto a Biotrans nylon membrane. Membranes were treated as described in the protocol for Southern transfer, colony and plaque lifts (page 65) and hybridized to the *cyt-2* probe (2.1 kb HindIII fragment). Colonies that gave a strong positive signal in the hybridization reaction were used to inoculate 5 ml

of LB-amp-tet media and cultures were grown to saturation (22 hours). The recombinant plasmids were isolated from the cultures by a rapid miniprep procedure (page 43), digested with the restriction enzyme EcoRI, which cuts at the ends of the inserts, and subjected to electrophoresis. DNA was transferred from the gel onto a Biotrans nylon membrane and hybridized to the *cyt-2* probe. The largest size EcoRI inserts that gave positive signals in the hybridization reaction were ligated into the M13 mp19 vector for sequencing.

Southern transfer, colony and plaque lifts.

Southern transfers were performed by a modification of the procedure described by Southern (1979). After electrophoresis, gels containing high molecular weight DNA were placed in a 1/50 dilution of a concentrated HCl for 10 to 15 minutes with gentle agitation prior to denaturation. Otherwise gels were put directly in a denaturing solution (1.5 M NaCl, 0.5 M NaOH). Following 30 minutes of gentle agitation the denaturing solution was replaced with neutralizing solution (3 M sodium acetate pH 5.5) and gently agitated for another 30 minutes. After draining off the excess buffer, the gel was placed on top of a glass plate covered with two sheets of Whatman 3 MM paper, forming a bridge between two reservoirs containing 10x SSC (1.5 M NaCl, 0.15 M sodium citrate pH 7.0). Alternatively, the gel was placed on a sponge soaked in 10x SSC which was covered with a Whatman 3 MM paper. A piece of Biotrans nylon membrane was placed on top of the gel and covered with a sheet of Whatman 3 MM paper which was then covered with a stack of paper towels and a 1 kg weight. Transfer of DNA from the gel onto the membrane was carried out overnight. The membrane was then air-dried and baked in an oven at 80°C for 1 hour.

For colony and plaque lifts, the Biotrans nylon membrane was carefully placed on the agar surface and marked for later orientation of colonies or plaques. After 1 minute, the membrane was removed and placed sequentially, colony side up, on Whatman 3 MM paper saturated with 10% SDS for 3 minutes, denaturing solution (see above) for 5 minutes, and neutralizing solution (see above) for 5 minutes. The membrane was then air-dried for 30 minutes and baked at 80°C for 1 hour. Membranes from colony lifts were prewashed with a solution containing 50 mM Tris-Cl pH 8.0, 1 M NaCl, 1 mM EDTA, 0.1% SDS, at 42°C for 1 to 2 hours to remove any absorbed medium or loose bacterial debris, before hybridization was set up.

Restriction analysis and agarose gel electrophoresis of DNA

Restriction enzyme analysis of DNA was performed according to the suppliers instructions. Restriction endonucleases were purchased from Bethesda Research Laboratories (BRL), Inc. or New England BioLabs, Inc.

Restriction fragments were electrophoresed on 0.8% (w/v) submarine agarose gels in 0.1 M Tris-borate pH 8.3, 2 mM EDTA, containing ethidium bromide (0.5 µg/ml). DNA samples were made to 5% (v/v) glycerol before loading on a gel, and electrophoresis was carried out at a constant voltage, 25 to 100 Volts. The bands were visualized and photographed on a UV transilluminator.

Purification of DNA from agarose gels

DNA fragments were either recovered from agarose gels onto DEAE membrane NA-45 (Schleicher and Schuell, Inc.) or electroeluted as described by Maniatis *et al.* (1982). After electrophoretic separation of the DNA on an agarose gel, a strip of NA-45 was placed in an incision just ahead of the band of interest. Electrophoresis was continued until the DNA band disappeared into the membrane, as judged by ethidium bromide fluorescence using a UV lamp. The membrane was rinsed with low salt NET buffer (0.15 M NaCl, 0.1 mM EDTA, 20 mM Tris-Cl pH 8.0); the DNA was eluted by submerging the membrane in 150 to 250 µl of high salt NET buffer (1.0 M NaCl, 0.1 mM EDTA, 20 mM Tris-Cl pH 8.0) in a microcentrifuge tube and incubating at 65°C for 30 to 45 minutes with occasional swirling. The buffer was removed to a fresh tube and the membrane washed with another 50 µl of the buffer. The combined buffer fractions were extracted once with water-saturated phenol and once with chloroform/iso-amyl alcohol (24/1) (v/v) and the DNA recovered by ethanol precipitation.

Mitochondrial cytochrome spectra

Mitochondria for cytochrome spectra were isolated from mycelium of *N. crassa* grown in liquid Vogel's medium with supplements, as required. The culture was harvested by filtration through a Whatman filter paper in a Buchner funnel and rinsed with cold water. The mycelium was ground on ice using a mortar and a pestle with acid-washed sand and a minimal amount of the isolation buffer (15% sucrose, 10 mM Tris-Cl pH 7.2, 5 mM EDTA) until a smooth paste was formed. More isolation buffer was added to suspend the ground mycelium and the slurry was transferred to an SS-34 centrifuge tube. The sand and cell debris was removed by centrifugation in an SS-34 rotor (Sorvall) at 4°C for 10 minutes at 3,000 rpm. The supernatant was transferred to a clean tube and centrifuged at 4°C for 30 minutes at 13,000 rpm. The mitochondrial pellet was resuspended in 1 ml of 10 mM Tris-Cl pH 7.2, 5 mM EDTA and 1 ml of 5% deoxycholate in 10 mM Tris-Cl pH 7.2 was added. After mixing, the solution was equally divided into two Eppendorf tubes, centrifuged in a microcentrifuge for 10 minutes, and the supernatant transferred to two cuvettes.

Cytochrome spectra were obtained by a modification of the procedure of Bertrand and Pittenger (1969) using a Perkin-Elmer Model 559 or a Shimadzu UV-265 recording spectrophotometer. Scanning was performed in the range from 650 nm to 500 nm of the visible spectrum. Air-oxidized samples were scanned to obtain a base line, after which the scan was repeated with a sodium dithionite reduced sample and a reference sample fully oxidized by potassium ferricyanide. The α absorption peaks for cytochrome aa_3, cytochrome *b* and cytochrome *c* are at 608 nm, 560 nm and 550 nm, respectively (Bertrand and Pittenger, 1972).

In vitro translation

In vitro translation was performed using a rabbit reticulocyte system (BRL Inc.) programmed with *N. crassa* poly(A) RNA. The radioactive amino acid used in the system was L-[^{35}S]methionine (>800 Ci/mmol) (Amersham). The *in vitro* translation reaction was performed following the manufacturer's instructions. Incorporation of the radioactivity was determined by counting samples (2 to 15 µl) from each reaction on GFC filters (Whatman). The filters were placed in large volume (10 ml/filter) of cold 10% trichloroacetic acid (TCA) for 10 minutes, then washed three times with 5% (w/v) TCA and twice with 95% ethanol. The filters were dried and counted in a Beckman LS7500 liquid scintillation counter after addition of 5 ml of scintillation fluid (Aqueous Counting Scintillant, Amersham).

Pulse labeling of *N. crassa* cells and isolation of labeled proteins

Pulse labeling of *N. crassa* cells with L-[^{3}H]leucine was done as described by Zimmermann and Neupert (1983) with some modifications. *N. crassa* cultures were grown in liquid Vogel's medium containing appropriate supplements (Davis and de Serrès, 1970). 600 ml of the medium was inoculated with fresh conidia to a concentration of 2 x 10^6 conidia/ml and was incubated with vigorous shaking at 25° to 30°C. After 36 hours of incubation for the *cyt-2-1* mutant (NCN68) and 14 hours for a wild-type (NCN10), 100 ml of culture was removed from each flask for mitochondrial spectral analysis to monitor for contamination. To the remaining culture, L-[^{3}H]leucine was added to a concentration of 2 mCi/l. Labeling was allowed to proceed for 3 minutes at room temperature, after which a dose of unlabeled leucine (final concentration 10 mM) was added. At this point, and after selected times, aliquots of the cultures were removed and quickly poured into 2 volumes of ice-cold water containing 10 mM unlabeled leucine, and then harvested by filtration. The mycelial pads were immediately placed into mortars containing liquid nitrogen (N_2). The samples were ground to a powder with acid-washed sea sand (1 g per g wet weight of mycelium) under liquid N_2 in the presence of 3% SDS, 10 mM Tris-HCl pH 7.5, 2 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (3 ml per g wet weight of mycelium). After thawing, samples were transferred to Sorvall centrifugation tubes and centrifuged at 4,000 rpm for 10 minutes at 20°C. The supernatant was collected and centrifuged in a 50Ti rotor (Beckman) at 23,000 rpm for 15 minutes at 20°C. After boiling the supernatant for 5 minutes, samples were dialysed against 1% Triton X-100, 0.1% SDS, 0.3 M NaCl, 10 mM Tris-Cl pH 7.5, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride for 36 hours with two buffer changes. Following the dialysis samples were centrifuged in an Eppendorf microcentrifuge for 15 minutes at 4°C and the supernatant was collected. Samples were stored at -20°C.

Immunoprecipitation of apocytochrome *c*

Immunoprecipitation of apocytochrome *c* from the labeled proteins was performed as described by Hennig and Neupert (1983). Immunoglobulins from 50 µl of antiserum raised against apocytochrome *c* or/and holocytochrome *c* (a gift from Dr. W. Neupert, University of Munich) were bound to 8 mg of protein A-Sepharose (CL-4B) (Pharmacia) in the presence of 1 ml of triton buffer (10 mM Tris-Cl pH 7.5, 0.3 M NaCl, 5 mM EDTA, 1% Triton X-100) for 1 hour at 4°C. The protein A-Sepharose-immunoglobulin complex was washed once in triton buffer and then mixed with labeled proteins containing 1-2 x 10^6 cpm of incorporated ^3H radioactivity, as determined by TCA counts. After 3 to 12 hour incubation with gentle mixing at 4°C, the protein A-Sepharose was pelleted by centrifugation for 2 minutes in an Eppendorf microcentrifuge. The pellets were washed three times with triton buffer and twice with the same buffer lacking Triton X-100. The final pellet was suspended in 50 µl 100 mM Tris-Cl pH 8.0, 5% SDS, 2.5% β-mercaptoethanol, left at room temperature for a minimum of 15 minutes and then boiled for 5 minutes. The protein A-Sepharose was removed by centrifugation and the supernatant was electrophoresed on an SDS polyacrylamide gel.

Polyacrylamide gel electrophoresis of proteins

Electrophoresis of immunoprecipitates from pulse-labeling experiments was carried out on 15% (w/v) polyacrylamide gels (diluted from a 30% (w/v) acrylamide/bisacrylamide (29/1) (w/w) stock), 0.1 M Tris-Cl pH 8.0, 1% (w/v) SDS vertical slab gels (20 cm x 20 cm x 1.5 mm). Gels were run at 100 Volts (constant voltage) with 0.1 M Tris-Cl pH 8.0, 1% (w/v) SDS as the reservoir buffer. Prior to loading, 1/10 volume of loading dye (0.1 M Tris-Cl pH 8.0, 1% (w/v) SDS, 50% (v/v) glycerol, 0.01% (w/v) bromphenol blue) was added to each sample which contained 100,000 to 250,000 cpm of ^{3}H labeled, immunoprecipitated proteins. Electrophoresis was carried on until bovine cytochrome *c* (MW = 12,000), used as a marker, migrated 6 cm into the gel. Using a manual gel slicer (Tyler Research), the gel was sliced into 1.1 mm slices which were transferred individually to plastic scintillation vials containing 0.75 ml of 0.1 M Tris-Cl pH 8.0, 1% (w/v) SDS. The proteins were eluted by incubating the vials at 70° to 80°C for at least 6 hours. 5 ml of scintillation fluid (Aqueous Counting Scintillant, Amersham) were then added to each vial and mixed vigorously. Samples were counted in a Beckman LS7500 Scintillation Counter.

Results

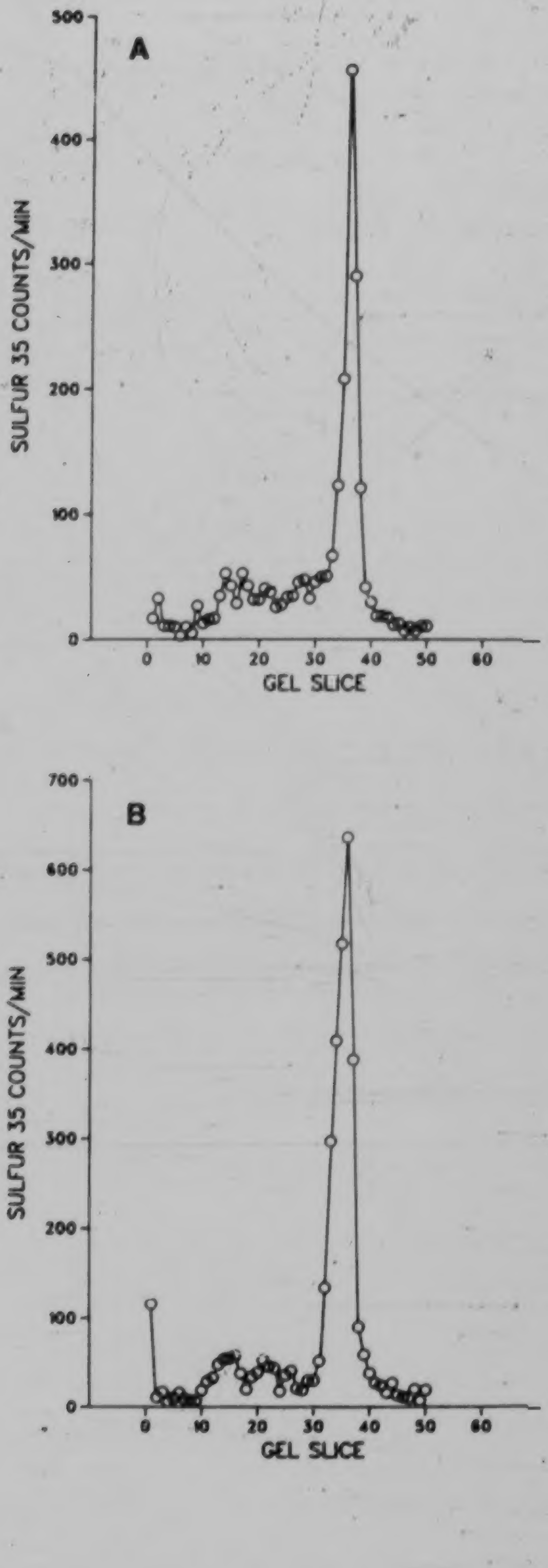
Detection of apocytochrome *c* in the *cyt-2-1* mutant

The deficiency of spectrally detectable cytochrome *c* in the *cyt-2-1* mutant described by Mitchell *et al.* (1953) and Bertrand and Collins (1978) was investigated by examining various steps in apocytochrome *c* synthesis and its transport into mitochondria. Previous experiments, by others in this laboratory, had shown that virtually no cytochrome ~~*c*~~ (neither holo- nor apo-) could be immunoprecipitated from either the mitochondria or the cytosol of *cyt-2-1* cells grown in the presence of [^{35}S]sulphate. To determine whether a translatable message for apocytochrome *c* was produced in the mutant, poly(A) mRNA from wild-type and *cyt-2-1* cells was translated in a rabbit reticulocyte system containing [^{35}S]methionine. The *in vitro* translation products were examined for the presence of immunoprecipitable apocytochrome *c*. As shown in Fig. 6, poly(A) mRNAs from both strains gave rise to immunologically detectable apocytochrome *c*, which indicates that the *cyt-2-1* mutant produces the mRNA for cytochrome *c*. The number of ^{35}S counts corresponding to the apocytochrome *c* immunoprecipitated from the *cyt-2-1* cells is approximately equal to or slightly higher than from wild-type cells, which indicates that the level of cytochrome *c* mRNA in the mutant is similar to the wild-type level.

Thus, it appeared that the protein was either inefficiently translated and/or rapidly degraded in the mutant. An attempt was made to distinguish between these possibilities using a pulse-chase experiment. Cultures of a

Figure 6. *In vitro* translation of apocytochrome *c* mRNA from wild-type and *cyt-2-1* cells.

Poly(A) RNA from wild-type (A) or *cyt-2-1* (B) cells was translated in the presence of [^{35}S]methionine, using rabbit reticulocyte lysates. In each case products of the *in vitro* translation, corresponding to 2 x 10^6 cpm for wild-type and 1.2 x 10^6 cpm for *cyt-2-1*, were treated with antibodies to apocytochrome *c* and the immunoprecipitates were resolved by SDS gel electrophoresis. Gels were sliced and the ^{35}S radioactivity in each slice was determined.

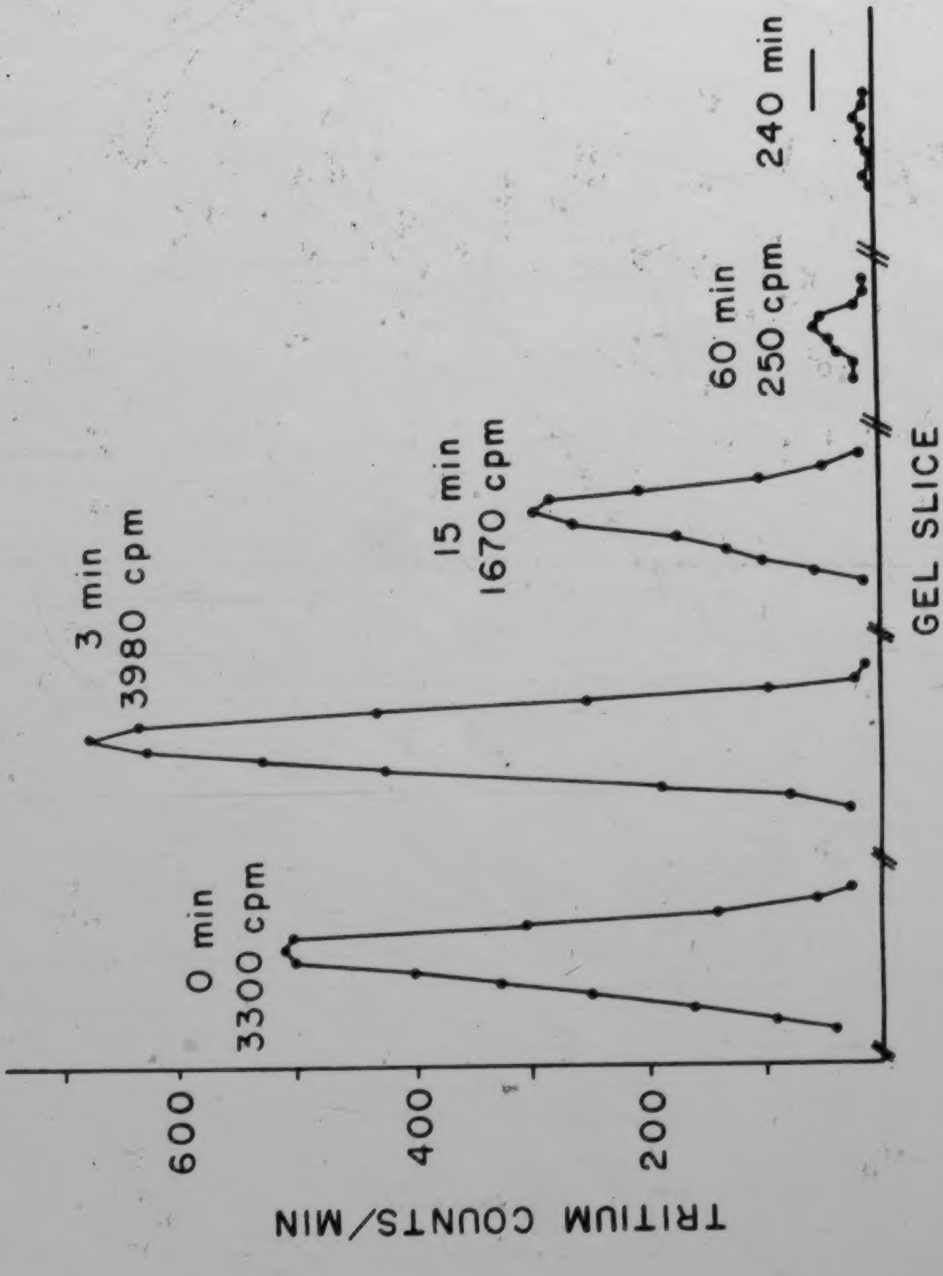

A
SULFUR 35 COUNTS/MIN
500
400
300
200
100
0
0
10
20
30
40
50
60
GEL SLICE
B
SULFUR 35 COUNTS/MIN
700
600
500
400
300
200
100
0
0
10
20
30
40
50
60
GEL SLICE

cyt-2-1 strain were labeled with [^{3}H]leucine for a brief period of time (as described in the Materials and Methods), after which an excess of unlabeled leucine was added to the culture. Samples were removed from the cultures at different times and total cytochrome *c* (both holo- and apo- forms) was immunoprecipitated from whole cell fractions. The immunoprecipitates were resolved by SDS gel electrophoresis and after slicing the gels, the radioactivity in each slice was determined. For each time point, the radioactivity in each gel slice corresponding to apocytochrome *c* was plotted as the number of counts per minute and the total radioactivity in each peak was compared. The results of this experiment show that the amount of immunoprecipitable apocytochrome *c* in the *cyt-2-1* cell decreases quite rapidly (Fig. 7). The amount of the pulse labeled product is significantly reduced 15 minutes after the addition of chase and almost completely disappeared after 60 minutes of chase. A control experiment with wild-type cells was not performed because earlier results showed that apocytochrome *c* is rapidly converted to its holo form and imported into wild-type mitochondria where it is stable for at least 30 minutes (Hennig and Neupert, 1981). Since no cytochrome *c* could be precipitated from the mitochondria of the *cyt-2-1* mutant (as shown by others in the laboratory) it can be concluded from these results that cytochrome *c* is translated in the mutant, but is then degraded in the cytosol. The slight increase in apocytochrome *c* that occurs in the sample taken 3 minutes after the addition of chase is likely due to a lag in the time of transporting the cold leucine into the cells and/or the completion of translation of nascent [^{3}H]leucine-labeled apocytochrome *c* chains during the initial moments of the chase.

After determining that apocytochrome *c* is translated in the mutant

Figure 7. Labeling of apocytochrome *c* in *cyt-2-1* cells in a pulse-chase experiment.

Each peak represents the total number of counts per minute associated with apocytochrome *c* that was immunoprecipitated from the *cyt-2-1* cells after different chase times. The total number of counts per minute in each peak and the time of incubation of the cells after adding the chase, prior to harvesting, are indicated.


TRITIUM COUNTS/MIN
600
400
200
0 min
3300 cpm
3 min
3980 cpm
15 min
1670 cpm
60 min
250 cpm
240 min
GEL SLICE

cells, its transport into the mitochondria was examined by others. The results of this analysis showed that *cyt-2-1* mitochondria are almost completely devoid of cytochrome *c* heme lyase activity, as well as deficient in high affinity binding sites for apocytochrome *c* (Nargang *et al.*, 1988).

Identification of the *cyt-2*$^+$ gene

The *cyt-2*$^+$ gene was isolated by sib selection and gene rescue of the *cyt-2-1* mutant from a *N. crassa* genomic library constructed in the pSV50 cosmid (Fig. 4). Spheroplasts of the *cyt-2-1* mutant were transformed with cosmid DNA isolated from a series of pooled clones as described in the Materials and Methods. Clones capable of complementing the *cyt-2-1* mutation gave fast-growing, benomyl resistant transformants against a background of slow-growing colonies, that had also been transformed to benomyl resistance. After 4 rounds of sib selection by transformation, a single cosmid, pSV50-7617, was identified as carrying the *cyt-2*$^+$ gene (Fig. 8). To confirm that the cloned DNA specifically restores the presence of cytochrome aa_3 and *c* in the mutant, cytochrome spectral analysis from six of the fast-growing *cyt-2-1* transformants obtained with this clone was performed. In each case, cytochromes were present in ratios comparable to those of wild-type mitochondria, indicating that the deficiencies of cytochromes aa_3 and *c* had been restored in the transformants (Fig. 9).

The chromosomal location of the cloned DNA in pSV50-7617 was determined by restriction fragment length polymorphism (RFLP) mapping in relation to various genetic markers (Metzenberg *et al.*, 1984; 1985). A standard set of progeny used for the mapping was obtained from the Fungal Genetic

Figure 8. Sib selection of the *cyt-2* gene.

In each of the four rounds of transformation the sib giving rise to fast-growing transformants is indicated by a plus sign. Sib number 7 which gave the largest number of fast-growing transformants in the first round of sib selection, was arbitrarily chosen for the next round of sib selection.

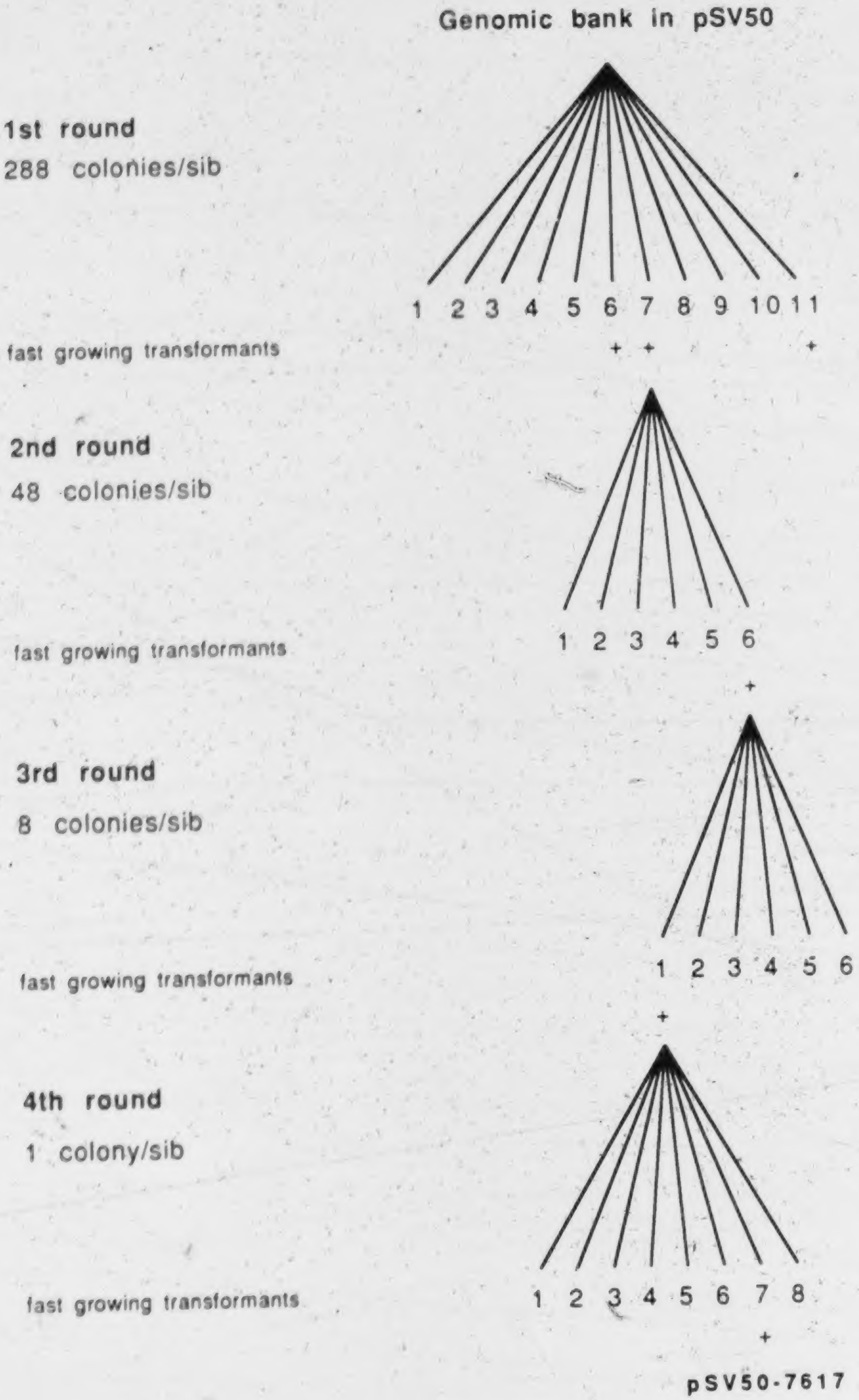
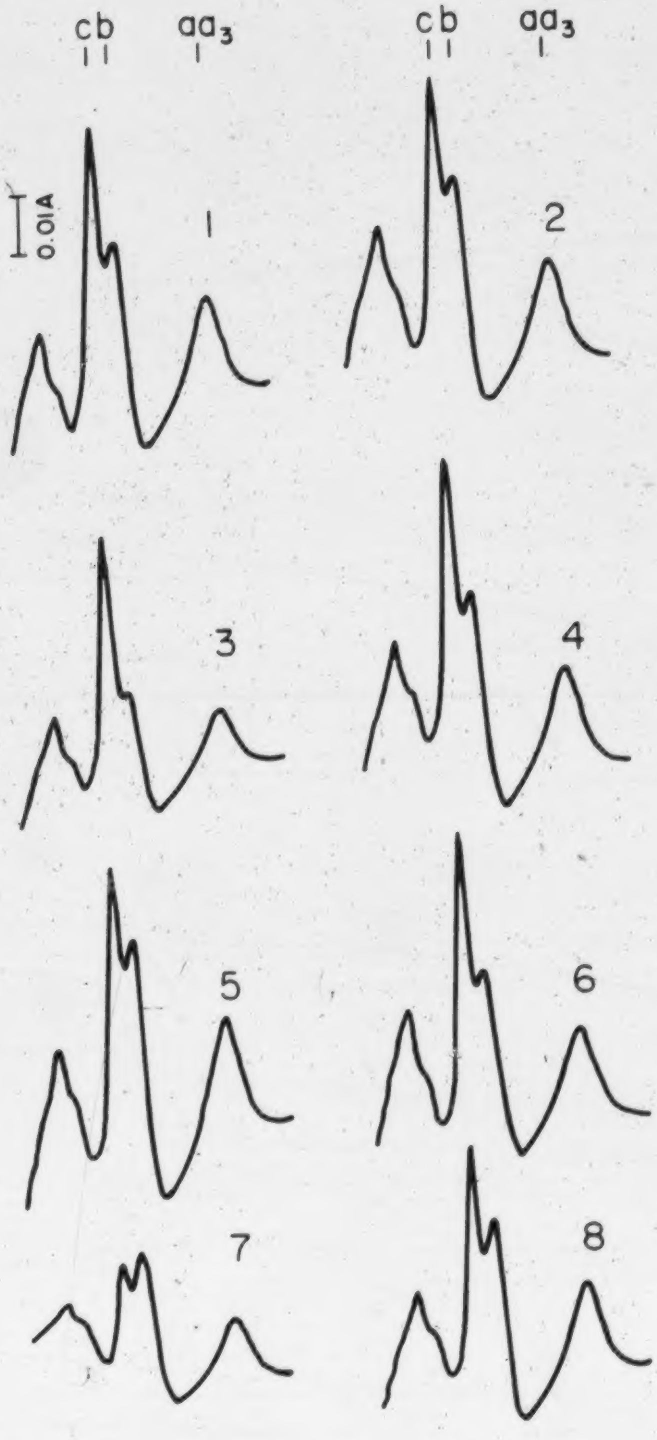

Genomic bank in pSV50
1st round
288 colonies/sib
1 2 3 4 5 6 7 8 9 10 11
fast growing transformants
+ + +
2nd round
48 colonies/sib
1 2 3 4 5 6
fast growing transformants
+
3rd round
8 colonies/sib
1 2 3 4 5 6
fast growing transformants
+
4th round
1 colony/sib
1 2 3 4 5 6 7 8
fast growing transformants
+
pSV50-7617

Figure 9. Mitochondrial cytochrome spectra of *cyt-2-1* transformants with pSV50-7617 .

Panel 1, wild-type; panels 2-7, fast-growing *cyt-2-1* transformants; panel 8, *cyt-2-1*. The positions of the α absorption bands of cytochromes aa_3 (608 nm), *b* (560 nm) and *c* (550 nm) are indicated.


c b
aa$_3$
c b
aa$_3$
0.01A
1
2
3
4
5
6
7
8

Stock Center. The parental strains in the cross were a laboratory strain RLM1-33*a* (Oak Ridge genetic background) carrying several genetic markers on different chromosomes, and strain Mauriceville-1c*A*, which has been recently isolated from nature and contains many restriction site polymorphisms when compared with the Oak Ridge strain. Southern analysis of DNAs from RLM1-33*a* and Mauriceville-1c*A* digested with various restriction enzymes revealed several restriction site differences between the two strains, that could be detected when the blot was probed with the pSV50-7617 cosmid (Fig. 10). The restriction endonuclease PstI was chosen for subsequent Southern analysis of DNAs isolated from 18 ascospore progeny of the cross, using the pSV50-7617 cosmid as a probe (Fig. 11). Each of the progeny displayed a hybridization pattern characteristic of either RLM1-33*a* (Oak Ridge) or the Mauriceville-1c*A* strain. The pSV50-7617 hybridization pattern for the individual progeny was compared with those of other markers to establish linkage (Metzenberg *et al.*, 1984). The analysis showed that the polymorphic fragment, homologous to the cloned insert, in 16 out of 18 progeny (11% recombination) segregates with the 5S rRNA-*50* gene, which is located on linkage group VI (Table 6). This is in agreement with previous genetic mapping data that showed the location of the *cyt-2-1* mutation to be on the left arm of chromosome VI (Bertrand *et al.*, 1977; Perkins *et al.*, 1982). However, because the *N. crassa* gene for β-tubulin, which imparts Bn^R, located on pSV50 also maps to linkage group VI, it was necessary to ensure that the polymorphism described above was within the region corresponding to the pSV50-7617 insert, and not to the *N. crassa* DNA within the pSV50 vector. Therefore, the Southern blot was reprobed with a 2.6 kb SalI fragment that contains the β-tubulin gene cut out of the pSV50 cosmid. This fragment hybridizes to a band of *ca*. 9.0 kb (Fig. 12), not related to the ones showing the restriction fragment length difference when the pSV50-7617

Figure 10. Detection of DNA restriction fragment length differences between two polymorphic strains of *N. crassa* using the pSV50-7617 clone as a probe.

A Southern blot containing pairs of DNAs from two polymorphic *N. crassa* strains, RLM1-33*a* (Oak Ridge, O) and Mauriceville-1c*A* (M) digested with various restriction enzymes was probed with the pSV50-7617 cosmid clone. Restriction enzymes used are indicated by numbers: (1) EcoRI, (2) HindIII, (3) BamHI, (4) PstI, (5) XbaI, (6) ClaI, (7) HinfI, (8) EcoRV, (9) BglII. Digestion with several enzymes, namely EcoRI, HindIII, BamHI, PstI, EcoRV and BglII resulted in detectable restriction fragment length differences between the two strains, when probed with the pSV50-7617 cosmid clone labelled with ^{32}P.

HindIII digested lambda DNA was used as a molecular weight standard; the fragment sizes are indicated on the right-hand side of the figure.

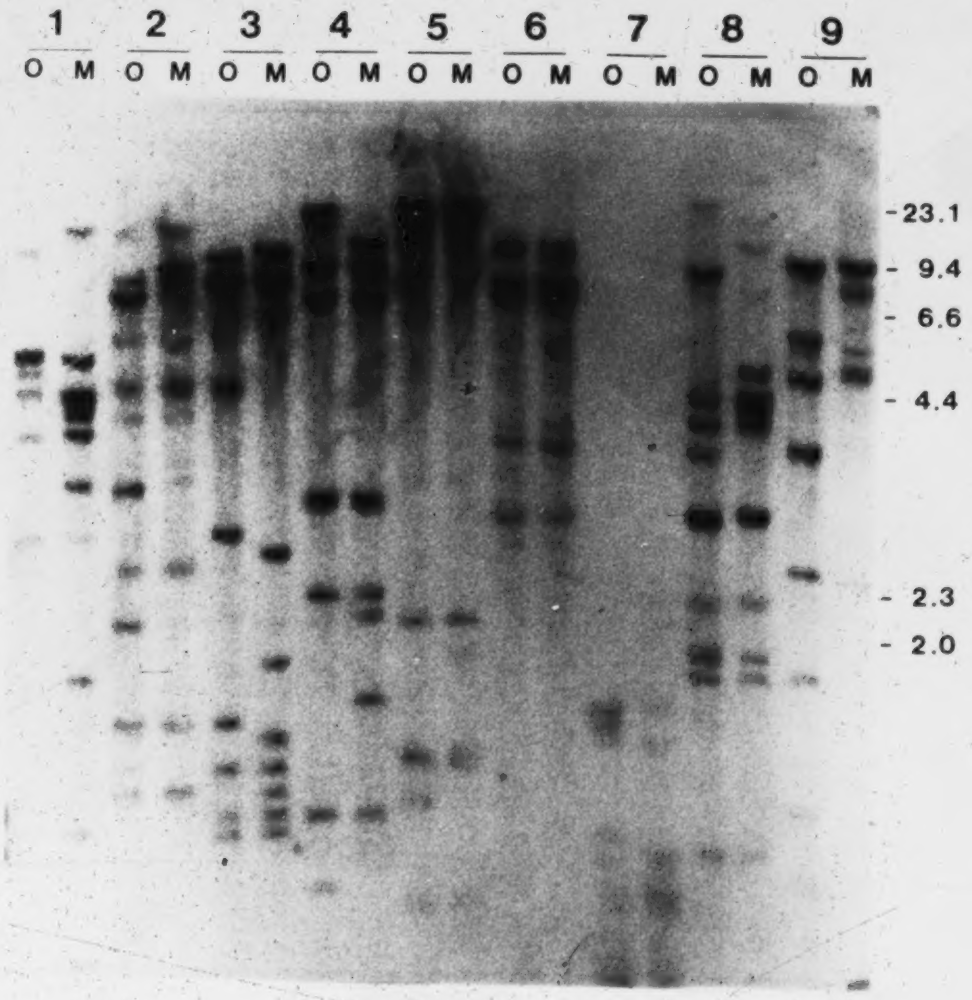

1 2 3 4 5 6 7 8 9
O M O M O M O M O M O M O M O M O M
23.1
9.4
6.6
4.4
2.3
2.0

Figure 11. Mapping the chromosomal location of the cloned cosmid DNA by restriction fragment length polymorphisms (RFLP).

A Southern blot was made from a gel containing PstI digested DNAs isolated from *N. crassa* mapping strains obtained from the Fungal Genetic Stock Center. The set contains two parental strains: RLM1-33*a* (Oak Ridge; indicated as O), and Mauriceville-1c*A* (indicated as M), as well as the progeny of the cross between them (indicated by numbers). The blot was probed with the pSV50-7617 cosmid clone labelled with ^{32}P.

Each of the progeny can be classified as having either the Oak Ridge (O) or the Mauriceville (M) restriction fragment pattern. Arrows indicate polymorphic restriction fragments that were used to determine Oak Ridge or Mauriceville genotypes of the progeny. "β" indicates the genomic fragment homologous to the HindIII-SalI fragment from the pSV50 cosmid vector carrying the β-tubulin gene.

The sizes of lambda HindIII restriction fragments electrophoresed on the same gel are indicated.

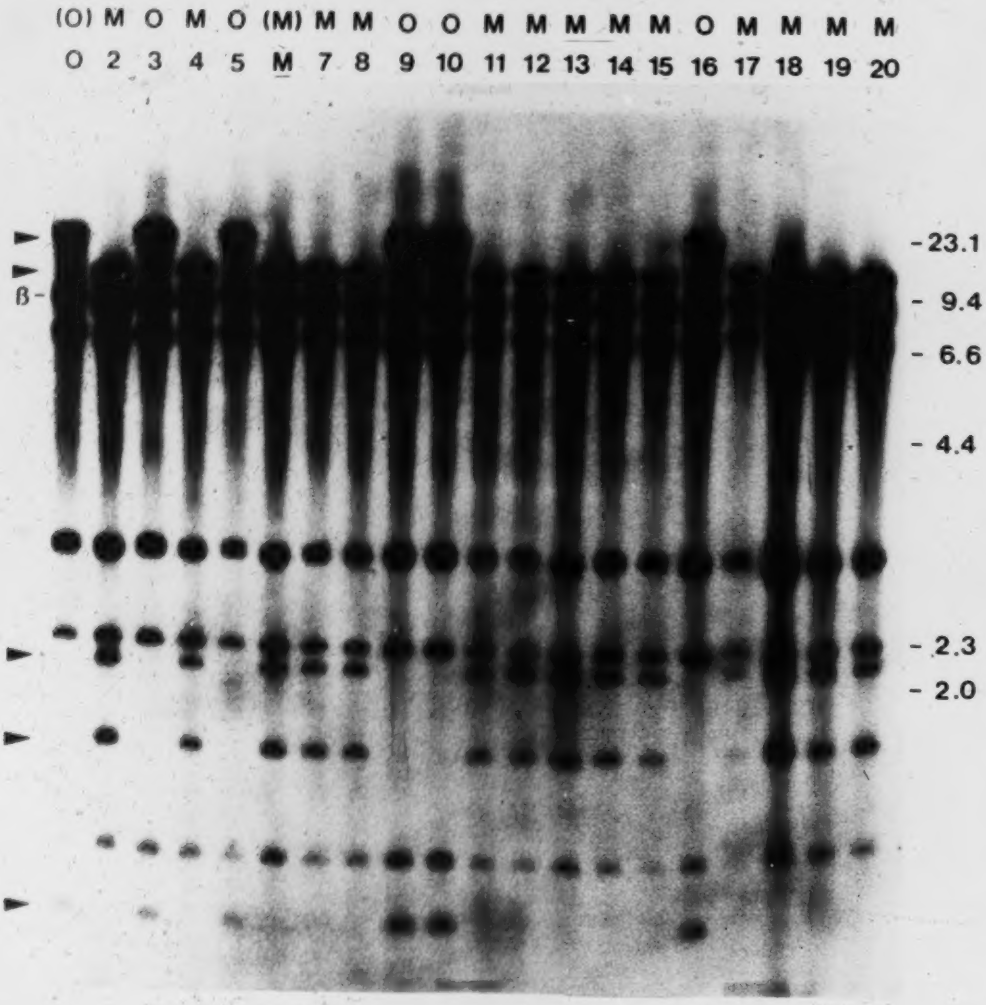

(O) M O M O (M) M M O O M M M M M O M M M M
O 2 3 4 5 M 7 8 9 10 11 12 13 14 15 16 17 18 19 20
β-
-23.1
- 9.4
- 6.6
- 4.4
- 2.3
- 2.0

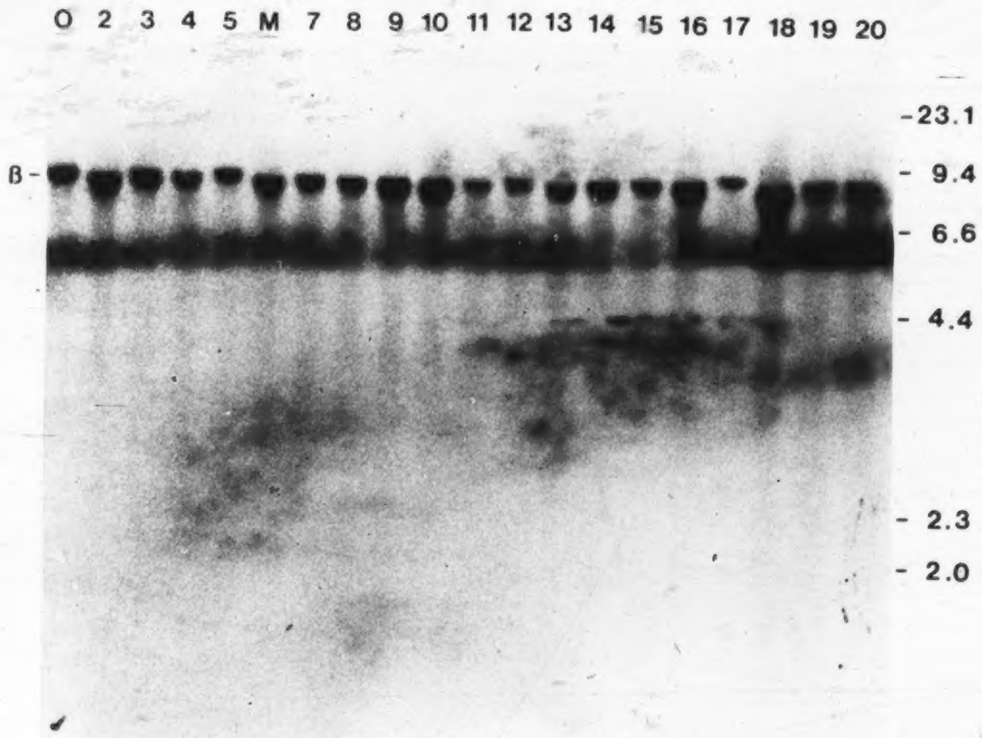
Table 6. Segregation of the 5S rRNA-*50* gene (Metzenberg *et al.*, 1984) and RFLP detected by pSV50-7617 in the progeny of the cross RLM1-33*a* x Mauriceville-1c*A*.

Abbreviations: O, RLM1-33*a* (Oak Ridge); M, Mauriceville-1c*A*; 7617, cosmid carrying the putative *cyt-2*$^+$ clone. (O) and (M) designate the parental strains of the cross.

lane # on Fig.11	FGSC strain	5S rRNA gene 50	7617 RFLP
1(O)	4411	(O)	(O)
2	4412	M	M
3	4413	M	O
4	4414	M	M
5	4415	O	O
6(M)	4416	(M)	(M)
7	4417	M	M
8	4418	M	M
9	4419	O	O
10	4420	O	O
11	4421	M	M
12	4422	M	M
13	4423	M	M
14	4424	M	M
15	4425	M	M
16	4426	O	O
17	4427	O	M
18	4428	M	M
19	4429	M	M
20	4430	M	M

Figure 12. Reprobing the blot used for mapping the pSV50-7617 insert with pSV50 cosmid vector.

The Southern blot used for mapping the chromosomal location of the pSV50-7617 cosmid clone (Fig. 11) was stripped and reprobed with the SalI fragment containing *N. crassa* DNA cut out of the pSV50 vector (Fig. 4). The band indicated as "β" is present in all the lanes on the autoradiograph in the same position. This band is a PstI fragment in the genomic DNA homologous to the HindIII-SalI fragment in the pSV50 cosmid containing the *N. crassa* β-tubulin gene.


O 2 3 4 5 M 7 8 9 10 11 12 13 14 15 16 17 18 19 20
β -
-23.1
- 9.4
- 6.6
- 4.4
- 2.3
- 2.0

cosmid is used as the probe (Fig. 11). This confirms that the observed RFLP is related to the insert in the pSV50-7617 clone and not to the β-tubulin gene.

Subcloning of the *cyt-2*$^+$ gene

The original cosmid clone was found to contain a chromosomal DNA insert of about 38 kb. The region of this DNA that contained the *cyt-2*$^+$ gene was deduced by cotransformation of the mutant with pSV50 plus various linear DNA restriction fragments generated from the original cosmid. This analysis revealed that a 9.6 kb BamHI fragment was capable of producing fast-growing transformants. This fragment was cloned into pUC19 vector to give plasmid pUCB2. Subsequently, transforming activity was found to be contained on a 2.1 kb HindIII fragment located within the larger BamHI fragment (Fig.13). This 2.1 kb HindIII fragment was subcloned into the bacterial vector pUC19 to give plasmid pMD6. The latter was isolated from *E. coli*, cut with various restriction enzymes, and inserted into appropriate cloning sites of the bacteriophage M13 mp18 or mp19 for DNA sequence analysis. Both strands of the 2.1 kb HindIII fragment were sequenced using the strategy demonstrated in Fig. 14.

5' end sequence extension

Analysis of the sequence (see below) revealed that only about 190 bp of sequence upstream from the predicted start site of the *cyt-2* open reading frame was included on the 2.1 kb HindIII piece. Therefore, to analyze flanking regions surrounding the coding sequence with respect to any regulatory

Figure 13. Partial restriction map of the 9.6 kb BamHI fragment containing the *cyt-2* gene.

The 9.6 kb BamHI fragment was subcloned from the original cosmid clone pSV50-7617. The restriction fragments indicated by bars were used for cotransformation with pSV50 of the *cyt-2-1* mutant cells. The results of the transformations, that is, the presence or absence of fast growing transformants, are shown as (+) or (-), respectively.

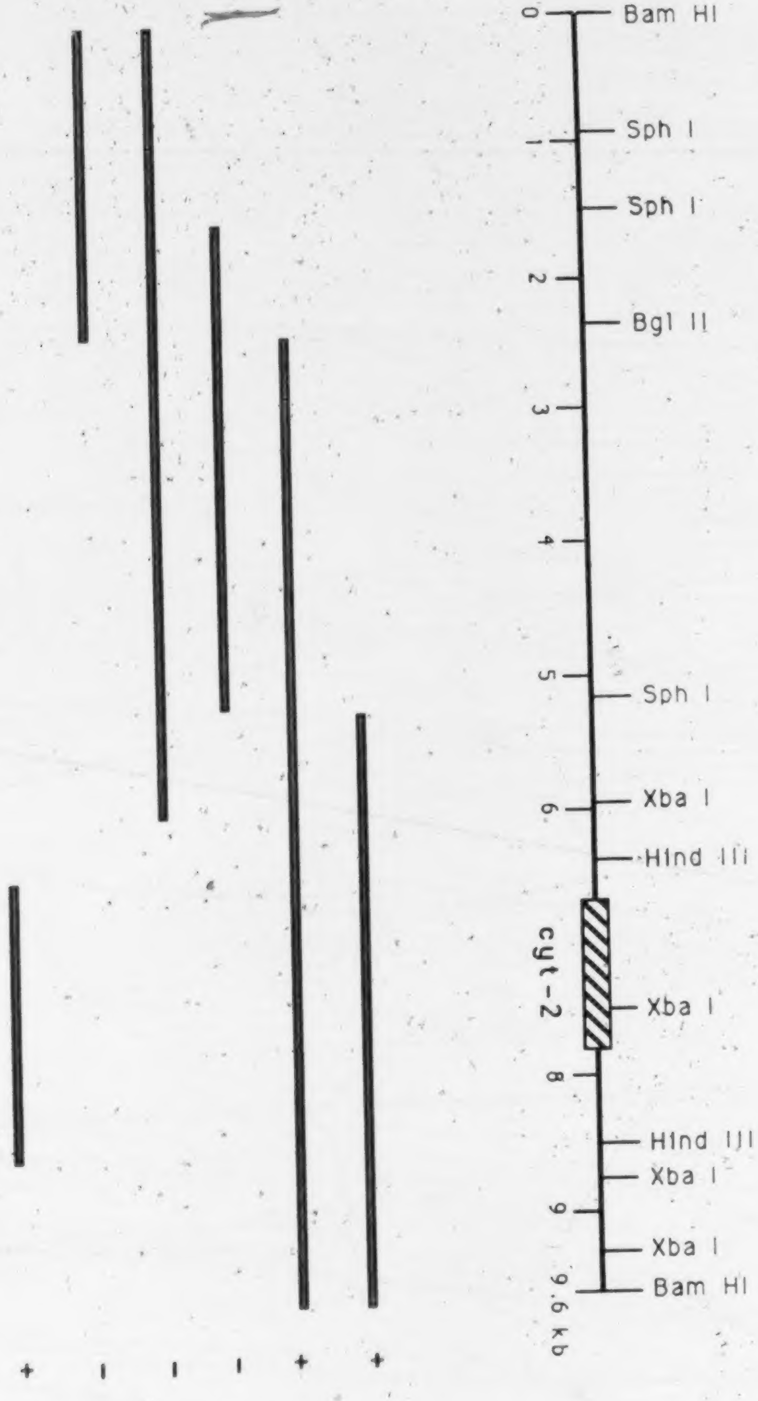
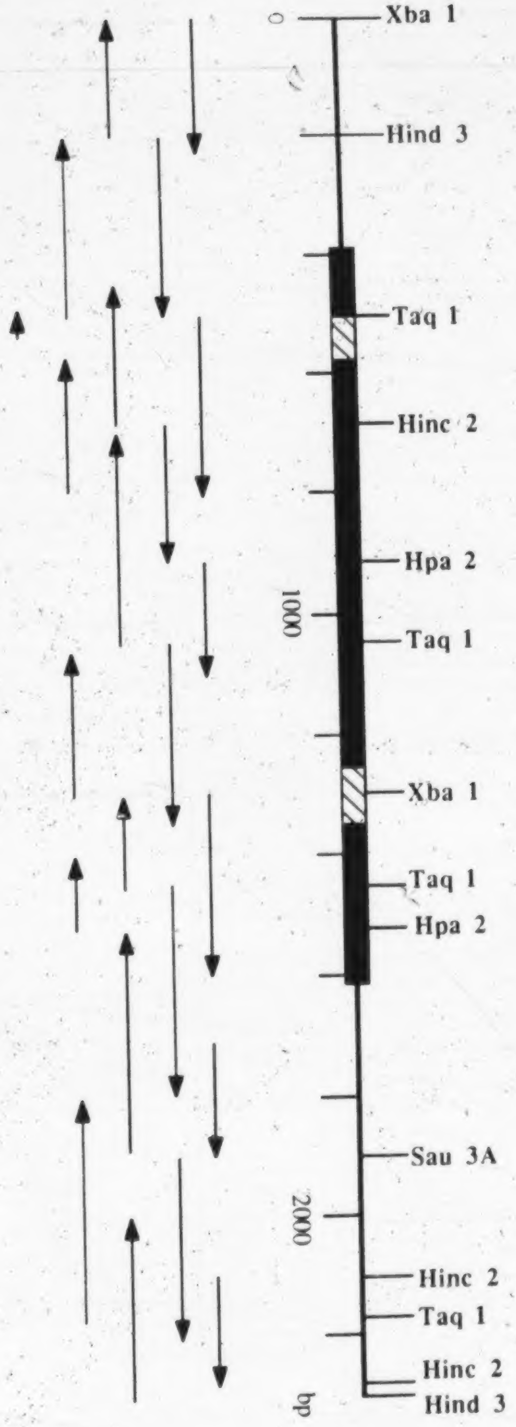

Bam HI
Sph I
Sph I
Bgl II
Sph I
Xba I
Hind III
Xba I
Hind III
Xba I
Xba I
Bam HI
cyt-2
0
1
2
3
4
5
6
8
9
9.6 kb
+
-
-
-
+
+

Figure 14. Partial restriction map and DNA sequencing strategy of the 2.3 kb XbaI-HindIII fragment containing the *cyt-2* gene.

The boxed area indicates the extent of the open reading frame; solid boxes represent exons, hatched boxes represent introns. Arrows indicate the length and direction of the sequence obtained from individual clones.


0
1000
2000
bp
Xba 1
Hind 3
Taq 1
Hinc 2
Hpa 2
Taq 1
Xba 1
Taq 1
Hpa 2
Sau 3A
Hinc 2
Taq 1
Hinc 2
Hind 3

elements that may control expression of the *cyt-2* gene, the sequence was extended at the 5' end of the *cyt-2* gene for another 200 nucleotides, up to the leftward XbaI site (Fig. 13). To do this, the larger 9.6 kb BamHI fragment carrying the *cyt-2* gene was digested with restriction enzyme XbaI and the resulting products run on an agarose gel. Both 1.5 kb and 1.3 kb XbaI fragments, each containing part of the *cyt-2* sequence, were isolated from an agarose gel and cloned into M13 mp18 or mp19 for sequencing. The 1.3 kb XbaI fragment, identified in the course of sequencing, as the one containing the 5' end of the gene, was sequenced on both strands through and past the HindIII site (Fig. 14). The extended sequence of the 5' untranslated region of the *cyt-2* gene is included in Fig. 15.

Analysis of the DNA sequence

The complete sequence of the 2.1 kb HindIII fragment and the XbaI extension is shown in Fig. 15. Analysis of the sequence revealed the presence of several open reading frames, some of which exhibited significant similarity to the amino acid sequence of yeast cytochrome *c* heme lyase (Dumont *et al.*, 1987). Using these regions of similarity and known *N. crassa* intron consensus sequences (Kinnaird and Fincham, 1983), it was determined that the coding sequence of the *Neurospora* gene is likely interrupted by two introns of 92 and 95 base pairs (Fig. 15). The sequences at the boundaries of both of these introns and their putative internal splice sites match the consensus sequences deduced from other *Neurospora* nuclear genes (Fig. 16). The positions of the introns was confirmed by sequencing a *cyt-2* cDNA clone isolated from a lambda ZAP cDNA library (see page 123). The predicted AUG start codon occurs

Figure 15. DNA sequence of the 2.3 kb XbaI-HindIII fragment containing the *cyt-2* gene.

The sequence of the entire HindIII fragment containing the *cyt-2* gene plus 200 additional upstream bases is shown. The first nucleotide in the putative start codon is designated as position 1. The numbering on the left-hand side of the figure refers to the nucleotide position with respect to the AUG codon. The numbering on the right side corresponds to the amino acid position in the predicted protein sequence. Gaps in the amino acid sequence indicate the presence of introns. Bases underlined once indicate the translation start sequence; bases underlined twice indicate sequences thought to be involved in intron splicing. Dotted lines underline the bases which demonstrate similarity to part of the UAS sequences of yeast that are implicated in heme mediated regulation of several genes (Table 9).

The bases deleted in the *cyt-2-1* mutant at position 1014 and 1015 are indicated as (●●); 5' ends of the two different *cyt-2* cDNA clones isolated are indicated by (▼), the larger arrowhead indicates the longer, 1.6 kb cDNA clone, the smaller arrowhead indicates the shorter, 1.4 kb cDNA clone; the 3' end of the *cyt-2* transcript is marked by (★).

```
-387    .         .         .         .         .         .         .         .         .
AGACGGTGAAAACCACATCGAAGTCTTGAGCTAACACACAGACCGATCCTGCGTGCAAGTCAATCCTGGTTGGGCGGGGCAGCCATCAAAGAAACGATGG

-287    .         .         .         .         .         .         .         .         .   ▼
CCCCAAGCGTGTCATGCCCGTCCGGCTTTATCCGCTCATATCGAAATTTCTCTTCGCCCATATTTTTTTTTATCTTCCATCCTCTTCTTGTTATTGAAGG

-187    .         .         .         .         .         .         .         .         .
AAGCTTACATTGTTCTCGCGCTATCCTTAGACTTTTTCCACCTCTGTTTGATATTAATTGAATCTACCTAACCCCAATTGTTTGTATATATCATCAACCG

- 87    .         .         .         .         . ▼       .         .         .  +1 .       .
TTCAAAGCAGTAGCCGGACTGCTCTTTTCTGCCCGACATTCACGGACATTTTCCCCCGTCAACGACAATCTTTTATAATCAGTCGCAATGGGTTGGTTTT
                                                                                     MetGlyTrpPheT   4

  14    .         .         .         .         .         .         .         .         .
GGGCTGACGGAAACGCTTCCGCCGCTGCGCCGGTCGTCCCTCCGTCGCATAAGGATCTTGCCGCCAGCGGTGCTGTTCCTCCTGTATGTCATCGTCGTCC
rpAlaAspGlyAsnAlaSerAlaAlaAlaProValValProProSerHisLysAspLeuAlaAlaSerGlyAlaValProPro                  32

 114    .         .         .         .         .         .         .         .         .
TCGAAACCCGCCCTCACCGGGGCCCTCCCAACTGCCAGTAGAGTCTGATCTAACCTCTTTGTCGCAACCATGTAGCCTTCCTGCCCTATGCACAACAAGA
                                                                           ProSerCysProMetHisAsnLysT  40

 214    .         .         .         .         .         .         .         .         .
CCATGGACGCCTTGAGCGCCCACAAGCCCGTAACACCAGCACCCGAACCGACTCCCGCCGCCGCTGCCCCTTCCAAGTGTCCCGTCAACCATGGCGCCAA
hrMetAspAlaLeuSerAlaHisLysProValThrProAlaProGluProThrProAlaAlaAlaAlaProSerLysCysProValAsnHisGlyAlaLy  73

 314    .         .         .         .         .         .         .         .         .
GGATACCCTCGCCGCTGCGGCCGCCGCCGTTGCGCCCAAGCAGCCCCAGCCAGAAAACCACCAACCCGCCGCCGCCTCCGAGCCTTCCTTCTTCTCCAAG
sAspThrLeuAlaAlaAlaAlaAlaAlaValAlaProLysGlnProGlnProGluAsnHisGlnProAlaAlaAlaSerGluProSerPhePheSerLys 107

 414    .         .         .         .         .         .         .         .         .
CTCAACCCCCTCAACTACATGTTCTCCTCCATCTCCCAAGAGCCTGCGCCCAACCAGGCCATCGCCCTCCCGACGGAGCGCGACCCCTCCTCCATCCCCA
LeuAsnProLeuAsnTyrMetPheSerSerIleSerGlnGluProAlaProAsnGlnAlaIleAlaLeuProThrGluArgAspProSerSerIleProL 140

 514    .         .         .         .         .         .         .         .         .
AGGGCACCGGCGACGGCAACTGGGAGTACCCTTCTCCCCAGCAGATGTACAACGCTCTCCTGCGCAAGGGCTACACCGACACCGACATTACCGCCGTGGA
ysGlyThrGlyAspGlyAsnTrpGluTyrProSerProGlnGlnMetTyrAsnAlaLeuLeuArgLysGlyTyrThrAspThrAspIleThrAlaValGl 173

 614    .         .         .         .         .         .         .         .         .
GAGCATGGTGGCCGTACACAATTTCCTGAACGAGGGCGCATGGAACGAGATTGTCGAGTGGGAGCGCCGGTTCGGCAAGGGTCTCATGCGCGGCTGGGAA
uSerMetValAlaValHisAsnPheLeuAsnGluGlyAlaTrpAsnGluIleValGluTrpGluArgArgPheGlyLysGlyLeuMetArgGlyTrpGlu 207

 714    .         .         .         .         .         .         .         .         .
ATTATGAAGCGCGGCGAGGAGAACGCGCCCATGATGCTGCGCCGGTTGGAGGCGCAGGAGAACGACCCCGAGCCCCAGCCGACGCTAATTCGGTTCCAGG
IleMetLysArgGlyGluGluAsnAlaProMetMetLeuArgArgLeuGluAlaGlnGluAsnAspProGluProGlnProThrLeuIleArgPheGlnG 240

 814    .         .         .         .         .         .         .         .         .
GCAGGCCGAAGGATATGACGCCCAAGGCGGCCTTGTTGCAGGTTTTGGGCAGGATAAACTCCAAGTATGCGTAAGTACAGTCCTCTTTTGTCTTTTTTTT
lyArgProLysAspMetThrProLysAlaAlaLeuLeuGlnValLeuGlyArgIleAsnSerLysTyrAl                              263

 914    .         .         .         .         .         .         .         .         .
TTCTAGATGAGGTTGGAAAAAGTAGCTTTTTTTTTGCTAACGATGTGACTTTACTCACTATCTAGCACCGAACCTCCCTTTGATCGCCATGACTGGTATG
                                                                  aThrGluProProPheAspArgHisAspTrpTyrV 275
```

```
1014
TCTCTCGCGACGAAAACGGCCAGAAGAAGGAGGTCCGCTACGTGATCGACTTCTACTCTGCTCCTCCTGAGCCCACTGGCGAGCCCGTTTTCTACCTCGA
alSerArgAspGluAsnGlyGlnLysLysGluValArgTyrValIleAspPheTyrSerAlaProProGluProThrGlyGluProValPheTyrLeuAs 308

1114
CGTCAGACCTGCCGTAACGGTCACCGGTGCTTGCGAGCGCTTGCTGAGGTGGGGTGGCGATGTGTGGTGGAAGGCTTCCGGCGGAGAGGTCCGGGAGCGG
pValArgProAlaValThrValThrGlyAlaCysGluArgLeuLeuArgTrpGlyGlyAspValTrpTrpLysAlaSerGlyGlyGluValArgGluArg 342

1214
GAGAGAAGCAAGTAAAGACGCAAGTCTGTGGTTAAGTGAGTAGTGGCAGCTCAGCTTTGCTGCGCAACACTTAAAAACTTTTGTGGAAAAAAAGAAAATG
GluArgSerLysEnd                                                                                      346

1314
AGTTGGGTGTACAGGCGTGGGTGGGGGCGTGGGCGTGCAAAGGATGTGATTGTGGACAAAGGCGGTTTGGATTTTCGGTTTAATCATCTGTTTTCAACGG

1414
CATATCATGCATATCATGGACGGCGTCTTGGGAACACGGGACACGGGTACGTGCTTTCAAGCCGTTCTTCTCTTTTACCATTTTCAATGTGGATCTATAC

1514
TCCCCTCATTTTTGAAGACCAACTATGTGTGCTGTTTTGAAATTCGGTGACACATTTTTTTCACATTGCTCAGCTTATCACGAATTTTGTCAAATTTGAC

1614
GACGGCCGTTATCTGCTGGTGAGCAGCACCATCGTCCATCTCTAATATCCACCACTTTATCAGCCTGACCCAACCTTGATGACCAGTTGGCAGGAAAGAA

1714
GAACATGCACGTTAACCGCACACTATCACTAGCGGCCGCAAGGACAAAGAAACAAGGCGCCTGTCGGTCCTGTCGAGCGTGATGCATGAAAGCCGTACAT

1814
ATGATTCTGGTTGAATTTCTGTTCTGCTTCCGGTGTCCCCTTAATGACCTTGAGCGAGCTATAGTTGCAAAAACAAGTTAACATAATGCTCTAGTGGCTC

1914
TTGTGGTCATGATCCAAGCTT
```

Figure 16. Comparison of the intron consensus sequences in the *cyt-2* gene with those found in other *N. crassa* genes.

Sequences compiled from: a) Kinnaird and Fincham (1983); b) Woudt *et al.* (1983); c) Arends and Sebald (1984); d) Legerton and Yanofsky (1985); e) Huiet and Giles (1986); f) Orbach *et al.* (1986); g) Kuiper *et al.* (1988); h) Roberts *et al.* (1988); i) Sachs *et al.* (1989); j) Stuart *et al.*, (1987); Bottorff, (1990); k) this study;

Gene	Intron number	Consensus sequences
am [a]	1	GTACGT------------GCTGAC-----17-----CAG
	2	GTAAGT------------GCTGAC-----13-----CAG
histone H3[b]		GTAAGT------------GCTAAC-----14-----CAG
histone H4[b]	1	GTAAGT------------ACTGAC-----17-----CAG
	2	GTACGT------------ACTAAC-----17-----CAG
ADP/ATP carrier[c]	1	GTATGT------------GCTAAC-----15-----CAG
	2	GTACGT------------GCTGAC----- 7-----TAG
his-3 [d]		GTAAGT------------GCTAAC-----10-----TAG
qa-1S [e]		GCACGT------------ACTAAC-----12-----CAG
tub-2 [f]	1	GTAAGT------------GCTGAC-----19-----TAG
	2	GTAAGT------------ACTAAC-----14-----CAG
	3	GTACGT------------GCTGAC----- 8-----CAG
	4	GTACGT------------GCTCAC----- 9-----CAG
	5	GTGCGT------------GCTAGC-----15-----CAG
	6	GTAAGT------------ACTGAG----- 8-----CAG
cyt-21 [g]	1	GTACGC------------GCTAAC-----17-----TAG
	2	GTACGT------------ACTGAC-----23-----CAG
con-10 [h]	1	GTATGT------------GCTAAC-----14-----CAG
	2	GTATGT------------ACCAAC-----14-----CAG
cya-4 [i]		GTAAGT------------ACTGAC-----17-----CAG
cyc-1 [j]	1	GTATGT------------TCTAAC-----14-----CAG
	2	GTACGT------------GCTAAC-----15-----CAG
cyt-2 [k]	1	GTATGT------------TCTAAC-----18-----TAG
	2	GTAAGT------------GCTAAC-----21-----TAG
N. crassa **consensus**		$\mathrm{GTA}^{A}_{C}\mathrm{GT}$----------$^{A}_{G}\mathrm{CT}^{A}_{G}\mathrm{AC}$---7-23---$^{T}_{C}\mathrm{AG}$

immediately after a conserved 8 nucleotide sequence, present prior to the initiation codon in other *Neurospora* genes (Fig. 17).

As shown in Fig. 15, the *cyt-2*$^+$ gene gives rise to a protein of 346 amino acids with a molecular weight of 38,150. A computer search for sequences similar to the *cyt-2* protein sequence in the EMBL Gene Bank was performed using the TFastaA program (Pearson and Lipman, 1988) which translates the DNA sequences in all six reading frame. The yeast cytochrome *c* heme lyase obtained the highest score in this search, with a value significantly higher than scores obtained by other polypeptides listed. The alignment of the *N. crassa cyt-2* gene product and the yeast cytochrome *c* heme lyase protein using the Microgenie^R program allowed me to detect regions of similarity between these proteins. The overall percentage identity of the two proteins, if the four big gaps of 9, 17, 31 and 28 amino acids in the alignment of the proteins are excluded in the calculations, is 43%. If conservative substitutions are also taken into account, the percentage similarity becomes 69%. The homology occurs in several blocks, which are separated by stretches of dissimilar sequence (Fig. 18).

Codon usage within the *cyt-2* gene is similar to that of other *Neurospora* genes (Table 7). Where possible, C in the third position of the codon tends to be favoured, and there is a bias against A in the third position (only 21 of 346 codons). However, the *cyt-2* gene does not show the very strong bias against A in the third position of codons that is demonstrated by highly expressed *Neurospora* genes such as *am* (1 codon ending in A of 454 total codons; Kinnaird and Fincham, 1983) or *tub-2* (4 codons ending in A of 448 total codons; Orbach *et al.*, 1986). Rather, *cyt-2* resembles weakly expressed genes like *trp-1* (40 codons ending in A of 760 total codons; Schechtman and

Figure 17. Alignment of the 8 nucleotide conserved region immediately preceding the ATG start codon of several *N. crassa* genes.

Sequences compiled from: a) Schechtman and Yanofsky (1983); b) Woudt *et al.* (1983); c) Kinnaird and Fincham (1983); d) Arends and Sebald (1984); e) Legerton and Yanofsky (1985); f) Munger *et al.* (1985); g) Newbury *et al.* (1986); h) Huiet and Giles (1986); i) Orbach *et al.* (1986); j) Stuart *et al.* (1987), Bottorff, (1990); k) Kuiper *et al.* (1988); l) Paluh *et al.* (1988); m) Roberts *et al.* (1988); n) Fu *et al.* (1989); o) Sachs *et al.* (1989); p) this study.

Gene	Conserved sequence
trp-1 [a]	CAATCACA ATG
histone H3 [b]	CCATCACA ATG
histone H4 [b]	ATATCAAA ATG
am [c]	CCTTCAAA ATG
ADP/ATP carrier [d]	ATATCACA ATG
his-3 [e]	AAAACACC ATG
MT [f]	CTATCAAA ATG
pyr-4 [g]	CAGCCAAC ATG
qa-1S [h]	CCGCCATC ATG
tub-2 [i]	CGGTCAAG ATG
cyc-1 [j]	CAGTCAAA ATG
cyt-21 [k]	GGTCCAAC ATG
cpc-1 [l]	CAGTCAAC ATG
con-10 [m]	TCGTCAAC ATG
cys- 3 [n]	ATGGCACA ATG
cya-4 [o]	CCGCCACC ATG
cyt-2 [p]	CAGTCGCA ATG
N. crassa **consensus**	$\mathrm{CX}^{G}_{A}\mathrm{TCA}^{AA}_{CC}$ ATG

Figure 18. Alignment of the *cyt-2* open reading frame and yeast cytochrome *c* heme lyase (CCHL).

The MicroGenie^R Alignment program was used to detect regions of similarity between the *cyt-2* open reading frame and cytochrome *c* heme lyase (CCHL) from *S. cerevisiae*. Minor adjustments to the alignment were made by inspection. Amino acid residues are shown in single-letter code. Spaces indicate gaps introduced to optimize alignment. Identical residues are indicated by solid bars; conservative amino acid substitutions are indicated by two dots. Families of amino acids considered to represent conservative changes are: G, A, S, T and P; I, L, V and M; D, E, N and Q; H, R and K; F, W and Y (Dayhoff *et al.*, 1978). Regions of high similarity are indicated by boxes with numbers above them. Two regions that may be involved in binding heme (see page xx) are labeled as HRD (heme-responsive domain) (Pfeifer *et al.*, 1989).

```
                   1                               NAD                              NAD
N.crasa  cyt-2  MGWFWADGNASAAAPVVPPSHKDLAASGAVPPPSCP MHNKTMDALSAHKPVTPAPEPTPAAAAPSKCPVNHGAKDTLAAAAAA  83
                |||||||  :::         ||:::::: : ::|| ||                 |:::::::::||||  |  |
yeast    CCHL   MGWFWADQKTTG         KDIGGAAVSSMSGCPVMH                 ESSSSSPPSSECPVMQGDND         50

                                                                                    2
         cyt-2  VAPKQPQPENHQPAAASEPSFFSKLNPLNYMFSSISQEPAPNQAIALPTERDPSSIPKGTGDGN  WEYPSPQQMYNALLRKG  164
                                       ::|||| |   ::    | | : || :|  ||||| ::|:|  ||||||||||||::|||
         CCHL                          RINPLNNMPE LAASKQPGQKMDLPVDRTISSIPK SPDSNEFWEYPSPQQMYANMVRKG  132

                                       3
         cyt-2      YTDTDITAVESMVAVHNFLNEGAWNEIVEWERRFGKFLMRGWEIMKRGEENAPMMLRRLEAQENDPEPQRTLIRFQGRP  243
                     ::    |||||| |||||||| |:|::|||:                            :  :::  || |::| |:|
         CCHL   KIGGSGEVAEDAVESMVQVHNFLNEGCWQEVLEWEK                            PHTDESHVQPKLLKFMGKP  163

                                              4                               5
         cyt-2  KDMTPKAALLQVLGRIN SKYATEPPFDRHDWYVSRDEN      GQKKEVRYVIDFYSAPPEPTGEPVFYLDVRPAV TVTG  318
                  ::|:|  : : |::  |::: | ||||||| | | |       :  ||||||:|||::| :  | | | :|||||: ::
         CCHL   GVLSPRARWMHLCGLLFPSHFSQELPFDRHDWIVLRGERKAEQQPPTFKEVRYVLDFYGGPDDENGMPTFHVDVRPALDSLDN  246

         cyt-2  ACERLLRWGGDVWWKASGGEVRERERSK  346
                | :|: |:   :   :|::
         CCHL   AKDRMTRFLDRMISGPSSSSSAP       269
```

Table 7. Codon usage in *cyt-2* compared with other *N. crassa* genes.

Numbers represent the occurrences of a particular codon which is used to specify a given amino acid in the analyzed genes. (a) *trp-1* (Schechtman and Yanofsky, 1983); (b) *am* (Kinnaird and Fincham, 1983); (c) *cyt-2* (this study); (d) *tub-2* (Orbach *et al.*, 1986).

aa	codon	trp-1	am	cyt-2	tub-2	aa	codon	trp-1	am	cyt-2	tub-2
Phe	TTT	7	1	2	0	Ser	TCT	14	3	3	6
	TTC	15	17	8	25		TCC	20	21	13	24
Leu	TTA	0	0	0	0		TCA	1	0	0	0
	TTG	10	1	6	2		TCG	8	1	1	0
Leu	CTT	21	13	1	6	Pro	CCT	11	3	13	2
	CTC	26	22	7	25		CCC	20	10	19	17
	CTA	2	1	1	0		CCA	5	0	2	0
	CTG	18	1	4	1		CCG	4	0	6	0
Ile	ATT	11	6	4	4	Thr	ACT	5	8	2	6
	ATC	33	12	4	9		ACC	22	8	8	17
	ATA	0	0	1	0		ACA	2	0	1	0
Met	ATG	15	9	11	20		ACG	5	0	4	0
Val	GTT	11	14	4	10	Ala	GCT	19	15	10	13
	GTC	36	19	9	23		GCC	37	36	23	16
	GTA	3	0	3	0		GCA	5	0	2	0
	GTG	13	0	4	3		GCG	7	1	7	1
Tyr	TAT	6	0	2	1	Cys	TGT	0	1	1	0
	TAC	10	15	7	13		TGC	10	5	2	7
Ter	TAA	1	1	1	1	Ter	TGA	0	0	0	0
	TAG	0	0	0	0	Trp	TGG	5	8	10	4
His	CAT	5	0	3	4	Arg	CGT	12	8	0	10
	CAC	13	10	4	6		CGC	12	9	10	11
Gln	CAA	7	0	2	3		CGA	0	0	0	0
	CAG	23	15	10	19		CGG	3	0	5	0
Asn	AAT	4	1	1	1	Ser	AGT	4	0	0	0
	AAC	20	22	15	24		AGC	12	3	4	1
Lys	AAA	1	0	0	0	Arg	AGA	4	0	2	0
	AAG	43	29	18	13		AGG	6	0	3	2
Asp	GAT	16	3	5	9	Gly	GGT	18	31	5	21
	GAC	24	12	11	12		GGC	34	23	15	16
Glu	GAA	3	0	5	1		GGA	7	0	2	0
	GAG	48	36	21	38		GGG	3	0	0	1

Yanofsky, 1983). By this criterion *cyt-2* can be considered to be expressed at a low level. This is in agreement with the observations on the levels of *cyt-2* specific transcripts (see below).

Three characteristic elements are found in the *cyt-2* sequence immediately upstream from the HindIII site. These 18-mers that start at positions -66, -206 and -238, resemble yeast regulatory sequences present in the 5' flanking sequences of the iso-1-cytochrome *c* gene (*CYC1*), a gene for catalase T (*CTT1*) and a gene for cytochrome *c* heme lyase (*CYC3*) (Table 8). The yeast sequences have been implicated in the heme mediated control of the expression of these genes (Guarente *et al.*, 1984; Spevak *et al.*, 1986; Dumont *et al.*, 1987). No sequences have been shown to function as UAS in *Neurospora* as yet, however, similar sequences were also found in the *cyc-1* gene encoding the *N. crassa* cytochrome *c* (Bottorff, 1990; Table 8).

Northern hybridization analysis of the *cyt-2* transcript.

To examine expression of the *cyt-2* gene, poly(A) mRNA was isolated from a wild-type strain of *N. crassa* and from the *cyt-2-1* mutant. The RNAs were electrophoresed on a denaturing gel and transferred onto nylon membrane. M13 strand specific probes were used to identify the transcribed DNA strand. A probe from the 1.1 kb HindIII-XbaI fragment of the gene, consisting of only the strand that is complementary to the predicted coding sequence in the nontranscribed strand, detects a single transcript (Fig. 19A, lane 2). A probe specific for the opposite strand does not detect a transcript (Fig. 19A, lane 1). Both the *cyt-2-1* and wild-type genes give rise to *cyt-2* specific transcripts that appear to be identical in size (Fig. 19B). The amount

Table 8. UAS-like sequences upstream of the *cyt-2* coding sequence.

UAS-like sequences upstream of the *N. crassa cyt-2* and *cyc-1* gene (Bottorff, 1990) compared with UAS sequences of the *CYC3* (Dumont *et al.*, 1987), *CYC1* (Guarente *et al.*, 1984) and *CTT1* (Spevak *et al.*, 1986) genes of yeast. The position of the 5' nucleotide of the UAS sequences refers to its distance from the translation initiation codon of the gene. The data on yeast genes was originally compiled by Dumont *et al.* (1987).

Gene	Position of the 5' nucleotide	Sequence
cyt-2	-238	CTCTTCGCCCATATTTTT
	-206	CTCTTCTTGTTATTGAAG
	- 66	CTCTTTTCTGCCCGACAT
cyc-1	-160	CTCTTGGTTTGCAACTTT
	-146	CTTTTTTGTTCCTACTAT
CYC3	-232	CTCTTGCGCGGCGTGGGA
	-170	CTCTTTTTATCGACTTTT
CYC1	-271	CTCTTTGGCCGGGGTTTA
	-228	CTCTTTGGCGAGCGTTGG
CTT1	-437	CTCTCCTGCGTGCTTTCA
	-388	CTCTTTTTCAAGGGGATC

Figure 19. Northern analysis of the *cyt-2* transcript.

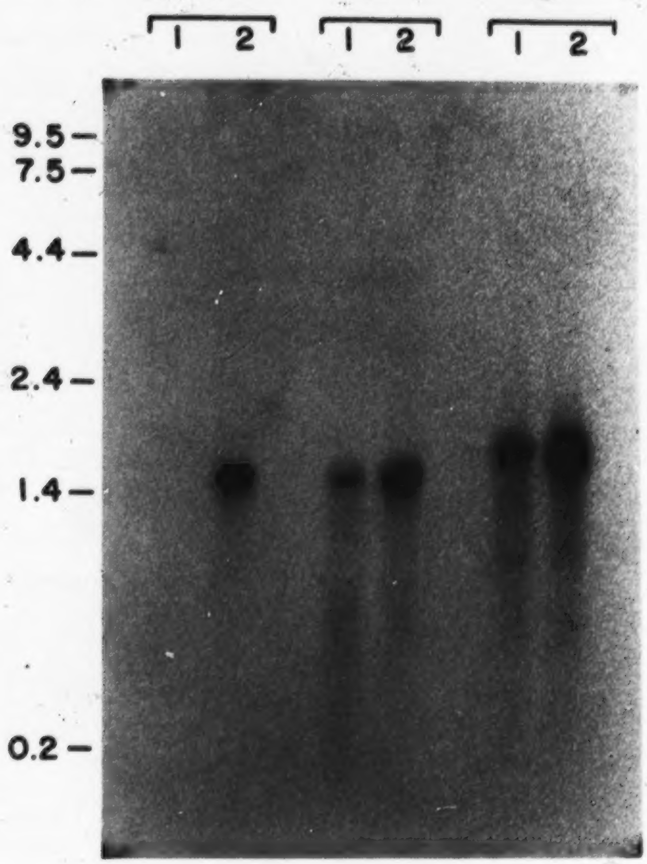
Poly(A) RNAs isolated from wild-type and *cyt-2-1* cells were electrophoresed, blotted and hybridized with *cyt-2* probes.

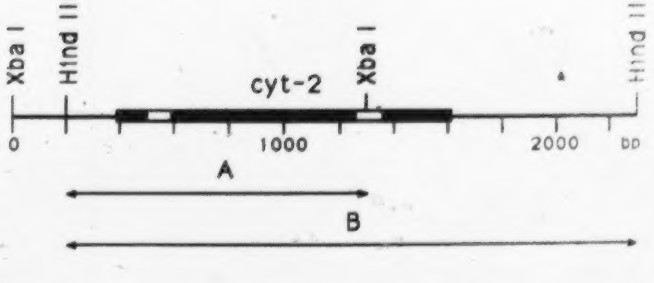
Panel A. Each lane contains 10 µg of poly(A) RNA isolated from cells of a wild-type strain. In lane 1 the blot was hybridized with a single-stranded probe, derived from the 1.1 kb HindIII-XbaI fragment (A; shown below), that is identical with the coding strand and would not hybridize to the predicted *cyt-2* mRNA sequence. In lane 2 the single-stranded probe was derived from the same fragment of DNA, but consisted of a strand complementary to the predicted mRNA from the *cyt-2* gene.

Panel B. Each lane contains 10 µg of poly(A) RNA isolated from either *cyt-2-1* (lane 1) or wild-type (lane 2) cells. The probe used was the 2.1 kb HindIII fragment containing the *cyt-2* gene (B, shown below).

Panel C. Each lane contains 2 µg of poly(A) RNA isolated from either *cyt-2-1* (lane 1) or wild-type (lane 2) cells. The probe used was a 2.6 kb SalI fragment carrying the β-tubulin gene isolated from the pSV50 plasmid (Fig. 4).

The sizes of RNA ladder fragments are indicated on the side of the autoradiograph.


A
B
C
1
2
1
2
1
2
9.5
7.5
4.4
2.4
1.4
0.2


Xba I
Hind III
cyt-2
Xba I
Hind III
0
1000
2000
bp
A
B

of this transcript appears to be lower in the mutant than in the wild-type strain. However, control experiments using a probe for β-tubulin mRNA on the same preparations of poly(A) mRNA (Fig. 19C) revealed a ratio similar to that observed using the *cyt-2* specific probe (Fig. 19B). The differences in message levels seen in the two strains are probably a reflection of the amount of rRNA remaining in the poly(A) mRNA preparations from the two strains.

The specific activities of the probes used in Fig. 19B and 19C were approximately equal (1.15 x 10^9 cpm/μg for the 2.6 kb β-tubulin probe, and 1.3 x 10^9 cpm/μg for the 2.1 kb *cyt-2* probe). However, the signal observed for the β-tubulin message from both the mutant and wild-type strain is much stronger than that for the *cyt-2* message, despite the fact that 5-fold more RNA was loaded on the gel that was examined with the *cyt-2* specific probe. Thus, although the autoradiographs are not strictly quantitative, the level of heme lyase transcript appears to be much lower than that of the β-tubulin gene. This is in agreement with the prediction about low level of expression of *cyt-2* gene based on the codon usage (see above). In yeast the cytochrome *c* heme lyase transcript is also present at low levels, about 1% of the level of *CYC1* mRNA (Dumont *et al.*, 1987).

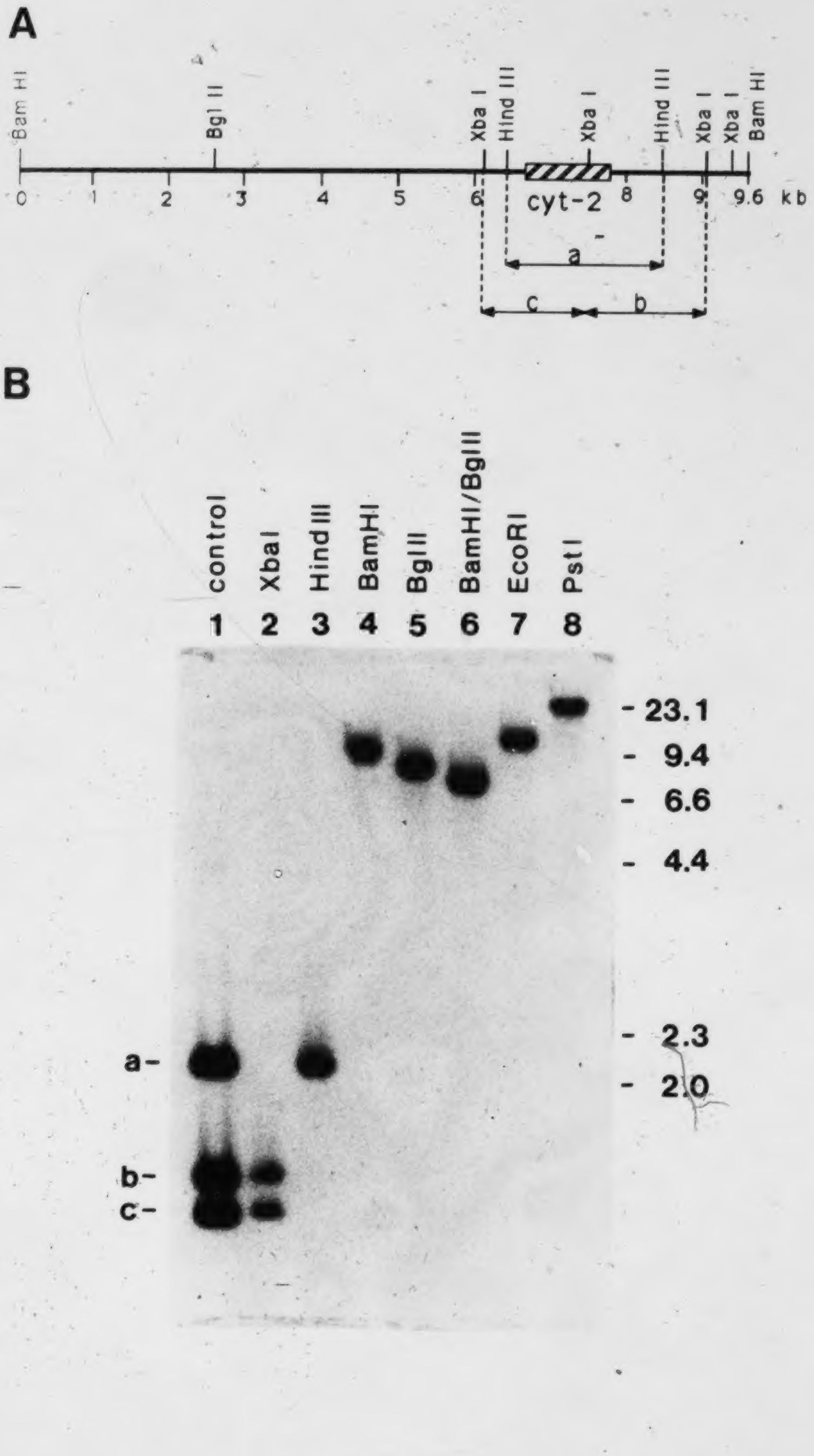
Cloning and sequencing of the *cyt-2-1* allele

In order to determine whether the *cyt-2-1* allele contained a gross alteration in its DNA sequence, DNA isolated from the mutant strain was digested with various restriction enzymes. The digests were electrophoresed, blotted to nylon membrane, and probed with the HindIII fragment containing the *cyt-2*$^+$ gene (Fig. 20A). This analysis revealed that the mutant allele was

Figure 20. Southern analysis of *cyt-2-1* DNA digested with various restriction enzymes.

Panel A. A partial restriction map of the 9.6 kb BamHI fragment from the pUCB2 plasmid, containing the *cyt-2*$^+$ gene.

Panel B. Lanes 2 through 8 show a Southern blot of *cyt-2-1* DNA digested with various restriction enzymes and probed with the 2.1 kb HindIII fragment containing the *cyt-2* gene labeled with ^{32}P. Lane 1 (control) shows three DNA fragments from a wild-type strain, containing a whole or a part of the *cyt-2* gene. The two XbaI fragments, 1.5 kb (b) and 1.3 kb (c) are derived from the plasmid pUCB2 digested with XbaI enzyme. 5 ng of the 2.1 kb HindIII (a) was added to the pUCB2 XbaI digest in lane 1 before loading the sample on a gel.


A
Bam HI
Bgl II
Xba I
Hind III
Xba I
Hind III
Xba I
Xba I
Bam HI
0
1
2
3
4
5
6
cyt-2
8
9
9.6 kb
a
c
b
B
control
XbaI
HindIII
BamHI
BglII
BamHI/BglII
EcoRI
PstI
1
2
3
4
5
6
7
8
23.1
9.4
6.6
4.4
2.3
2.0
a-
b-
c-

comparable in size to the wild-type allele (Fig. 20B, lanes 1, 2 and 3, 4). In addition, since there is only one band present in each lane and hybridization occurred only with the bands predicted by the restriction map of the wild-type gene (Fig. 20, lanes 2, 4-8), it is likely that only one copy of the *cyt-2* gene exists in *N. crassa*.

The *cyt-2-1* mutant allele was isolated from a partial DNA library of the *cyt-2-1* strain. To construct this library, HindIII restriction fragments ranging in size from 1.8 to 2.3 kb were isolated by gel electrophoresis and cloned into the bacterial vector pKGS. About 2,000 clones were screened by colony hybridization with the 2.1 kb HindIII fragment containing the *cyt-2*$^+$ gene used as the probe. Plasmid DNAs isolated from two clones that gave a positive response were further examined by restriction enzyme analysis and were found to contain a 2.1 kb HindIII insert. When the cloned *cyt-2-1* version of the gene was used to transform the *cyt-2-1* strain, no rescue of the phenotype was observed. Various restriction fragments from one of these recombinant plasmids (pKGS-5) were subcloned into M13 mp18 and mp19 for DNA sequence analysis.

The complete sequence of the 2.1 kb *cyt-2-1* mutant clone was compared with the *cyt-2*$^+$ gene sequence and found to contain only one change, a deletion of two bases (CT) from the predicted *cyt-2* coding sequence (Fig. 21). The two base pair deletion occurs at position 1014 of Fig. 15, changing the reading frame for 21 amino acids before generating an early stop codon (TGA) near the carboxy terminal end of the putative polypeptide (Fig. 22). Thus, the mutant protein contains only 297 amino acids compared to the 346 in the wild-type protein.

Figure 21. DNA sequence gel showing the site of the 2 base (CT) deletion in the *cyt-2-1* relative to the wild type sequence.

The bases deleted from mutant are circled in the wild type sequence. Because it is impossible to determine which CT pair in the three (CT)-repeat is missing in the *cyt-2-1* sequence, the first CT pair has been arbitrarily indicated as being deleted. The position of the mutation in the DNA sequence is indicated on Fig. 15.

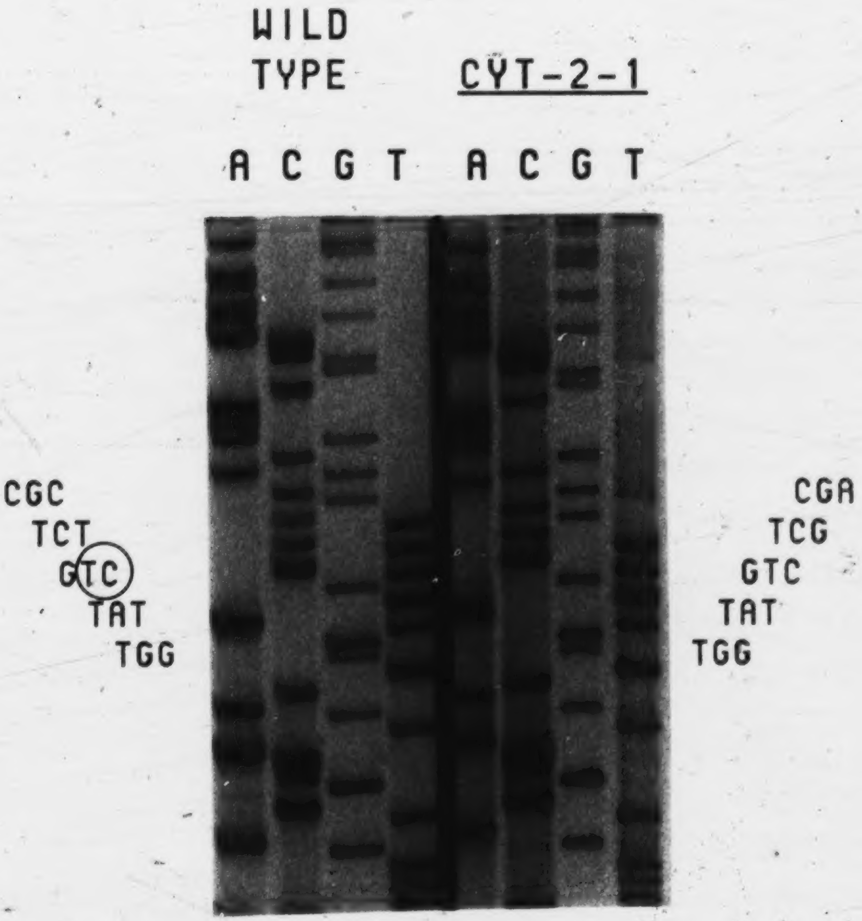

WILD TYPE
CYT-2-1
A C G T A C G T
CGC
TCT
GTC
TAT
TGG
CGA
TCG
GTC
TAT
TGG

Figure 22. Alteration of the amino acid sequence in the *cyt-2* open reading frame as a result of the *cyt-2-1* mutation.

Nucleotide and corresponding amino acid sequence of the carboxy terminal portion of wild-type and the *cyt-2-1* allele are shown. The position of the *cyt-2-1* mutation, 2 base (CT) deletion, is indicated. Extended regions of similarity with the yeast cytochrome *c* heme lyase are boxed; numbers above the boxes refer to the blocks of similarity indicated on Fig. 19.

wild-type

```
                     4                                               5
...P  F  D  R  H  D  W  Y  V  S  R  D  E  N  G  Q  K  K  E  V  R  Y  V  I  D  F  Y  S  A  P  P  E  P
...CCCTTTGATCGCCATGACTGGTATGTCTCTCGCGACGAAAACGGCCAGAAGAAGGAGGTCCGCTACGTGATCGACTTCTACTCTGCTCCTCCTGAGCCC

                  5
   T  G  E  P  V  F  Y  L  D  V  R  P  A  V  T  V  T  G  A  C  E  R  L  L  R  W  G  G  D  V  W  W  K
   ACTGGCGAGCCCGTTTTCTACCTCGACGTCAGACCTGCCGTAACGGTCACCGGTGCTTGCGAGCGCTTGCTGAGGTGGGGTGGCGATGTGTGGTGGAAG

   A  S  G  G  E  V  R  E  R  E  R  S  K
   GCTTCCGGCGGAGAGGTCCGGGAGCGGGAGAGAAGCAAGTAA
```

cyt-2-1

```
                     4
...P  F  D  R  H  D  W  Y  V  S  R  R  K  R  P  E  E  G  G  P  L  R  D  R  L  L  L  C  S  S
...CCCTTTGATCGCCATGACTGGTATGTCTCGCGACGAAAACGGCCAGAAGAAGGAGGTCCGCTACGTGATCGACTTCTACTCTGCTCCTCCTGA
                               |
                               TC deleted
```

Isolation of the *cyt-2* cDNA clone from a λ ZAP library.

In order to confirm the positions of the two introns within the *cyt-2* gene, as well as the 3' and possible 5' ends of the transcript, *cyt-2* cDNA clones were isolated and sequenced. The cDNA clones were isolated from a *N. crassa* cDNA library constructed in bacteriophage lambda ZAP. This library was generously provided by Dr. Matthew Sachs (Stanford University, Stanford, California). About 300,000 lambda ZAP clones were screened by plaque lifts and hybridization with the 2.1 kb HindIII fragment containing the *cyt-2*$^+$ gene. Seven clones which gave positive signals in the hybridization reactions with the genomic *cyt-2* clone, were further purified and submitted to the excision process (Fig. 5). The resulting circular DNA molecules (phagemids which contain a functional bacteriophage f1 origin and are capable of replicating autonomously) carried the cDNA inserts. The size of the inserts was determined by performing restriction analysis and Southern hybridization. The three largest cDNA inserts, with estimated sizes of 1.6 kb, 1.6 kb and 1.4 kb, were subcloned into M13 mp18 and mp19 phages for sequencing. One of the 1.6 kb clones was completely sequenced, the other two clones were sequenced only at their 3' and 5' ends.

The DNA sequence through the exon/intron boundaries confirmed the positions of the two introns whose presence in the genomic sequence was predicted on the basis of sequences characteristic of the 5' and 3' ends of *Neurospora* introns (Fig. 15). No other differences were found between the sequence of the isolated *cyt-2* cDNA clone and its genomic equivalent. Sequence at the 3' ends of all the cDNA clones revealed that the *cyt-2* transcript ends 319 nucleotides past the TAA stop codon at position 1544 of the genomic *cyt-2* sequence (Fig. 15). The sequence TATGTGTGCTG, immediately precedes a poly(A) tail and does not exhibit any similarity to the sequences prior to poly(A) tails of other *Neurospora* genes.

The 5' ends of the two independently isolated 1.6 kb cDNA clones start 191 nucleotides upstream from the AUG codon (Fig. 15). If the 5' end of the cDNA clone indeed corresponds to the transcription start point, the resulting transcript would be 1.55 kb in length. This is in agreement with the estimated size (1.57 kb) of the *cyt-2* transcript detected in the Northern hybridization analysis (Fig. 19), assuming a short poly(A) tail. The surrounding sequence ATTGAAGGA does not match sequences proposed as a "capping" site for eukaryotic genes, except for an A (underlined), which is present at the 5' end of many *Neurospora* transcripts (Table 9).

The third of the cDNA clones, with an estimated size of 1.4 kb, starts just 35 nucleotides upstream from the initiator AUG codon (Fig. 15). The surrounding sequence at the 5' end of this transcript is TTTTCCCCC, with the nucleotide C marking the beginning of the putative mRNA. This could be a second transcription start site for a shorter transcript, however only 1.57 kb transcripts were detected in the Northern hybridization analysis (Fig. 19). Thus it seems more likely that the 1.4 kb transcript represents a premature termination in the cDNA synthesis by reverse transcriptase.

Primer extension of the 5' end of the *cyt-2* transcript

An attempt to determine the extent of the *cyt-2* transcript at its 5' end by primer extension analysis with reverse transcriptase was made. A synthetic 18-mer, homologous to a sequence within exon 1 (position 75 to 58 with respect

Table 9. Sequences surrounding 5' end of *N. crassa* nuclear transcripts.

The underlined sequences are the most likely transcription initiation points as determined by primer extension or S1 mapping. Sequences were compiled from: a) Alton *et al.* (1982); b) Kinnaird and Fincham (1983); Arends and Sebald (1984); d) Legerton and Yanofsky (1985); e) Munger *et al.* (1985); f) Kuiper *et al.* (1988); g) Roberts *et al.* (1988); h) Sachs *et al.* (1989); i) Fu *et al.* (1989); j) Fu and Marzluf, (1990); k) this study; l) Sures *et al.*, (1978).

Gene	Sequence	Position from AUG codon
qa-2 [a]	GTATAGT	- 88
	CTATTTT	- 92
am [b]	TGCTCAT	- 95
	TTCTCTT	-111
	CTCTCGTTCTTT	-124
ATP/ADP carrier[c]	TCTCCAT	- 48
his-3 [d]	TACTCCAT	-125
MT[e]	TCATCAAC	-129 (major)
	TCATCACT	- 81 (minor)
cyt-21 [f]	GTCCGCGC	- 87 (major)
	TCAGTGAG	-128 (minor)
con-10 [g]	TCAAGCAA	- 96
cya-4 [h]	GACTTCAC	-150
cys-3 [i]	GTATGGAGCG	- 30
nit-2 [j]	GAAAGCGCA	-287
cyt-2 [k]	TGAAGGA	-191 (cDNA)
	TCATATCG	-252
Eukaryotic consensus [l]	PyCATTCPu	

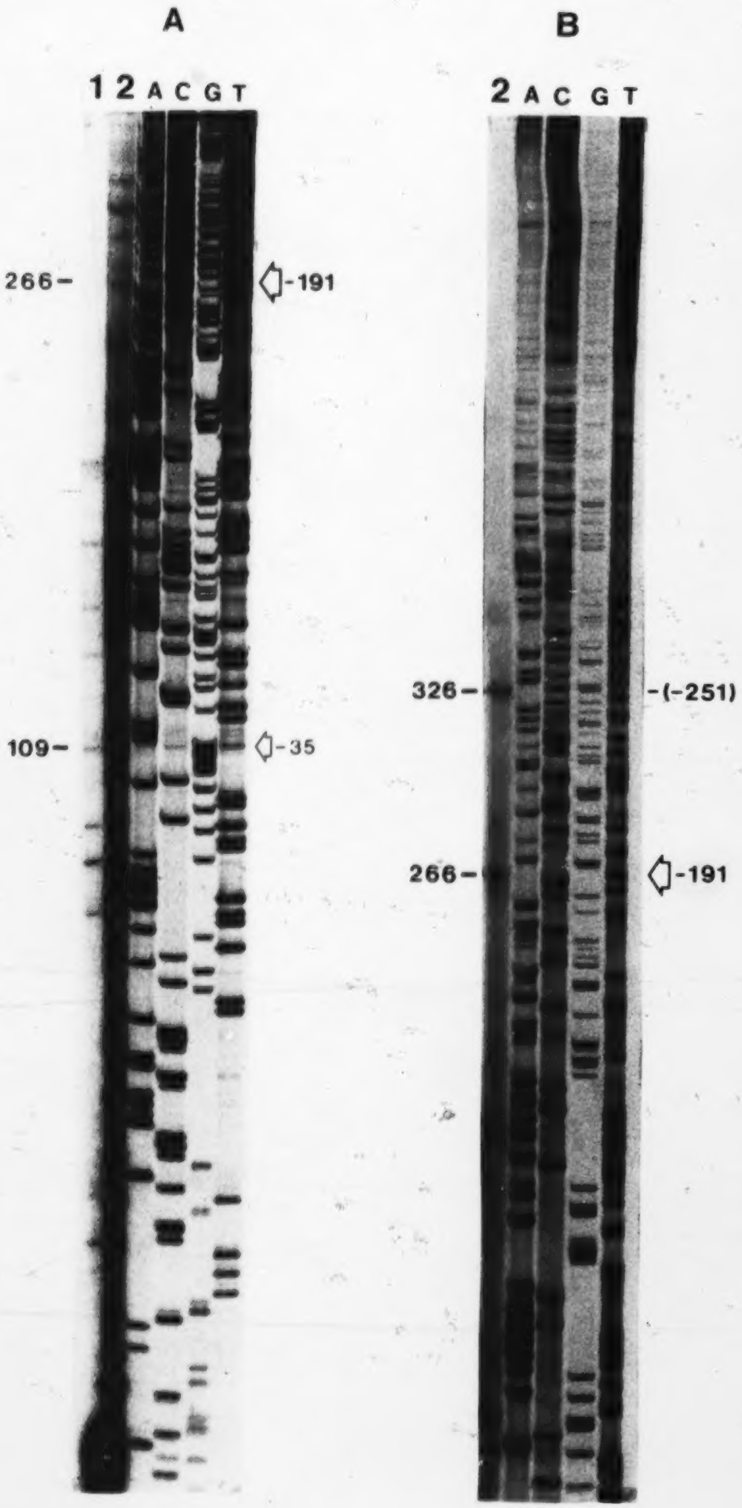
to the initiator AUG codon; Fig 15) was end labeled with [γ-^{32}P]dATP and annealed to poly(A) mRNA isolated from a wild-type strain of *N. crassa.* The primer extension reaction was performed with reverse transcriptase in the presence of all four dNTPs. The products of the reaction were resolved on a polyacrylamide denaturing gel together with the products of a sequencing reaction initiated by the same primer on an M13 clone containing the 1.6 kb *cyt-2* cDNA. Many bands appear in lanes 1 and 2 of the autoradiograph shown in Fig. 23A. Most of them probably represent products of premature termination by reverse transcriptase during the primer extension reaction. One such product, 109 nucleotides in length ends at a G (position -35 in the genomic sequence; Fig. 15), which corresponds to nucleotide C at the 5' end of the 1.4 kb cDNA clone. Another weaker band, 266 nucleotides in length, corresponds to position -191 in the genomic sequence (Fig. 15) which marks the beginning of the 1.6 kb cDNA clone. Several bands that appear on the autoradiograph past the indicated 266 nucleotides fragment (panel A, lane 2) suggest that the *cyt-2* transcript may in fact be longer than the isolated 1.6 kb cDNA clone.

The two primer extension reactions whose products are shown in Fig. 23A were performed with (lane 1) or without (lane 2) actinomycin D to prevent self-copying of the primer (Williams and Mason, 1985). To obtain better separation of the products of the sequencing reaction the gel in Fig. 23B was electrophoresed for a longer time (3 hours, compared to 1.5 hour for Fig. 23A). In this reaction actinomycin D, which also inhibits reverse transcriptase, was omitted to provide more cDNA products to generate stronger bands. Several bands appear in lane 2 on Fig. 23B. One of them is the fragment that is 266 nucleotides long, the same as shown in panel A, which

Figure 23. Primer extension analysis of *cyt-2* transcripts.

Panel A. Lanes 1 and 2 show cDNAs produced from a synthetic primer, (dGGCAAGATCCTTATGCAG), complementary to the sequence within exon 1, using wild-type poly(A) RNA (12 µg/lane) as a template. Primer extension reactions were carried out either in the presence (lane 1) or absence (lane 2) of actinomycin D. The cDNAs are aligned with dideoxy sequencing reactions using the same primer on an M13 clone containing the 1.6 kb *cyt-2* cDNA (page 127). Several bands are visible in each lane. Two bands, 109 and 266 nucleotides in length, are indicated. These bands correspond to the positions of the 5' ends of the two largest *cyt-2* cDNA clones isolated from the λ ZAP cDNA library (page 127). Arrows indicate nucleotides and their position in the genomic sequence (Fig. 15) at the 5' end of the two cDNA clones: a T (complementary to the A at position -191) at the 5' end of the larger, 1.6 kb cDNA clone (large arrow), and a G (complementary to the C at position -35) at the 5' end of the smaller, 1.4 kb cDNA clone (small arrow).

Panel B. Lane 2 show products of the primer extension reaction performed as before (lane 2, panel A), except that the products of the reaction were incubated with 5 µl of RNase A (100 µg/ml) and were separated further on a gel. The cDNAs are aligned with the same dideoxy sequencing reactions, as in panel A. The 266 nucleotide band that corresponds to the 1.6 kb *cyt-2* cDNA clone is indicated on the figure. Another higher band, corresponding to a fragment of 326 nucleotides in length could represent a longer *cyt-2* transcript that starts at position -251 in the genomic sequence (Fig. 15).


A
1 2 A C G T
266-
-191
109-
-35
B
2 A C G T
326-
-(-251)
266-
-191

corresponds to the 5' end of the 1.6 kb cDNA clone. The other band is 60 nucleotides longer and it ends at position -252 in the genomic sequence (Fig. 15). Again, this may indicate that some or all of the *cyt-2* transcripts are longer than the isolated cDNA clones. The first four nucleotides in the sequence TCATATC that surrounds the 5' end of this fragment are identical with a proposed consensus sequence for a eukaryotic "capping site", PyCATTCPu (Sures *et al.*, 1978). Similar sequences are found near the 5' end of the Neurospora *his-3* (Legerton and Yanofsky, 1985), MT (Munger *et al.*, 1985) and *con-10* (Roberts *et al.*, 1988) transcripts (Table 9). On the other hand it is possible that the primer is hybridizing nonspecifically and that this band represent products of the primer extension reaction with another mRNA. Considering the low level of expression of *cyt-2* mRNA, it is likely that most bands seen on the autoradiographs are due to priming at other sites in the poly(A) RNA mixture.

Thus, the analysis is inconclusive. Longer primers (30 to 40 nucleotides), located closer to the 5' end of the longest cDNA clone, should be used to make the data more definitive. Also, the conditions of the hybridization and primer extension reaction should be optimized by trying different primer to template ratios, different temperatures and higher concentrations of the nucleotides. In addition, S1 nuclease analysis of the *cyt-2* transcript should be performed independently of the primer extension reaction, since artifacts often arise in each of these analyses and therefore the results have to be confirmed by a different method.

Discussion

Several aspects of the *cyt-2-1* mutation of *N. crassa* have been elucidated in the course of this work. Studies of the cytochrome *c* deficiency in the *cyt-2-1* strain have shown that cytochrome *c* biosynthesis is altered at a post-translational step in the mutant. Analysis of poly(A) RNAs from *cyt-2-1* cells revealed that mRNA for cytochrome *c* is produced in the mutant and that it can be translated into apocytochrome *c* in an *in vitro* heterologous system. Using a pulse-chase experiment, it was determined that apocytochrome *c* is synthesized *in vivo* in *cyt-2-1* cells, but is then degraded in the cytosol. Further analysis of *cyt-2-1* mitochondria revealed that they are almost completely devoid of cytochrome *c* heme lyase activity (Nargang *et al.*, 1988). Attachment of the heme group to apocytochrome *c* by cytochrome *c* heme lyase is thought to induce a conformational change in the polypeptide, which drives its translocation across the mitochondrial membrane (Nicholson *et al.*, 1988; Dumont *et al.*, 1988). In the absence of cytochrome *c* heme lyase activity, apocytochrome *c* is unable to enter the mitochondria in *cyt-2-1* and appears to be rapidly degraded in the cytosol. Thus, it can be concluded that lack of cytochrome *c* heme lyase activity in *cyt-2-1* mitochondria is responsible for the absence of spectrally observed cytochrome *c* in the mutant (Nargang *et al.*, 1988).

The *cyt-2-1* mutant is analogous to the *cyc3* mutants of *Saccharomyces cerevisiae* which are also deficient in cytochrome *c* heme lyase (Dumont *et al.*, 1987). These mutants lack both forms of cytochrome *c* that occur in yeast, that is, iso-1- and iso-2-cytochrome *c* (Sherman *et al.*, 1965; Matner and Sherman, 1982). In *cyc3* mutant strains that exhibit gross deficiencies of both forms of holocytochrome *c*, apo-iso-2-cytochrome *c* was present in the cytosol, but apo-iso-1-cytochrome *c* could not be detected. The absence of apo-iso-1-cytochrome *c* is not caused by a transcriptional (Laz *et al.*, 1984) or translational (Dumont *et al.*, 1990) defect, but results from degradation of the polypeptide which, cannot be transported into mitochondria because it lacks cytochrome *c* heme lyase, (Dumont *et al.*, 1988; 1990). It was shown by pulse-chase experiments that the apo-iso-1-cytochrome *c* has a short half-life in heme lyase-deficient cells, whereas apo-iso-2-cytochrome *c* is relatively stable (Dumont *et al.*, 1990). By examining the stability of chimeric forms of apocytochrome *c* made up of a central portion from iso-2-cytochrome *c* inserted between the amino- and carboxy-termini of iso-1-cytochrome *c*, small internal regions from iso-2-cytochrome *c* were found to be responsible for the increased stability of the apoprotein (Dumont *et al.*, 1990). The replacement of as few as four residues in apo-iso-1-cytochrome *c* by the corresponding residues from apo-iso-2-cytochrome *c* is sufficient to substantially decrease the susceptibility of apo-iso-1-cytochrome *c* to degradation (Dumont *et al.*, 1990).

Dumont *et al.* (1990) postulate that the differential stability of the two isocytochromes *c* in yeast may be part of a regulatory process that increases the proportion of iso-2-cytochrome *c* under certain physiological conditions. As discussed earlier (see Introduction) the genes encoding the two iso- forms of cytochrome *c* in yeast, *CYC1* and *CYC7*, are inversely regulated by heme/oxygen at the transcriptional level. Under anaerobic conditions or during catabolite repression most of the cytochrome *c* in the cell consists of iso-2-cytochrome *c* (Sherman and Stewart, 1971). In partially repressed yeast cells, that is, in cells that have just exhausted fermentable substrates or that have been induced by oxygen after a period of anaerobic growth, higher levels of iso-2-cytochrome *c* are due to both differential transcription and different stability of the apoproteins. Under partially repressing conditions, synthesis of heme lyase or other components required for import of cytochrome *c* into mitochondria such as heme, may be repressed. As a result, transport of apocytochromes *c* into mitochondria may be less efficient. The proportion of apo-iso-2-cytochrome *c*, the more stable of the two apo- forms, would then be expected to increase in the cytosol. Consequently, apo-iso-2-cytochrome *c* would constitute a higher fraction of the cytochromes *c* that are eventually imported into mitochondria and converted into holocytochrome *c*. In support of this view, a *cyc3* mutant which is only partially defective in heme lyase, contains a small amount of residual holocytochrome *c* that is composed of mostly holo-iso-2-cytochrome *c* (Dumont *et al.*, 1990). In *Neurospora*, which is an obligate aerobe, this type of adaptation is unnecessary and only one cytochrome *c*, equivalent to yeast iso-1-cytochrome *c*, exists.

To determine the primary function affected by the *cyt-2-1* mutation, the *cyt-2*$^+$ gene of *N. crassa* was isolated from a genomic library by complementation of the cytochrome-deficient mutant. Cytochrome spectral analysis of the mitochondria from *cyt-2-1* cells transformed with the *cyt-2*$^+$ gene showed that both cytochromes, aa_3 and *c*, were restored. The idea that the *cyt-2*$^+$ gene was isolated from the library, rather than a suppressor gene, was supported by the results from the RFLP mapping of the cloned DNA. The results showed that the DNA capable of rescuing the *cyt-2-1* mutant was derived from chromosome VI, where the original mutation was mapped (Bertrand *et al.*, 1977; Perkins *et al.*, 1982). Further genetic characterization to demonstrate linkage to other markers on linkage group VI was not done since the mutant *cyt-2-1* allele was shown to contain a frameshift mutation directly affecting the gene rescued from the wild-type library. Taken together, these findings virtually eliminate the possibility that the clone which rescues the *cyt-2-1* phenotype is a suppressor of the *cyt-2-1* mutation.

DNA sequencing of a 2.1 kb HindIII fragment that restores a wild-type phenotype to *cyt-2-1* transformants, revealed the presence of an open reading frame that exhibits similarity to the amino acid sequence of yeast cytochrome *c* heme lyase (Dumont *et al.*, 1987). Since this enzyme was shown to be deficient in *cyt-2-1* mitochondria (Nargang *et al.*, 1988), it can be concluded that the *cyt-2* gene encodes the *N. crassa* cytochrome *c* heme lyase.

The *cyt-2* gene is interrupted by two small introns and encodes a polypeptide of 346 amino acids, with a molecular weight of 38,150. This polypeptide is considerably larger than the yeast heme lyase, an enzyme of 269 amino acids with a predicted molecular weight of 30,081 (Dumont *et al.*, 1987). Comparison of the sequences of the two proteins shows blocks of highly conserved amino acid sequences separated by less conserved sequences (Fig. 18). The conserved stretches of amino acids in the yeast and *Neurospora* cytochrome *c* heme lyase proteins may play an important role in the function or structure of the enzyme. This notion is supported by the finding that the *cyt-2-1* mutation destroys the reading frame of the gene prior to the last block of homology between the yeast and *N. crassa* cytochrome *c* heme lyase (Fig. 22). Thus, it seems likely that the mutation abolishes the activity of the enzyme, though it is conceivable that the lack of cytochrome *c* heme lyase activity in *cyt-2-1* mitochondria (Nargang *et al.*, 1988) is simply due to an inability to transport the altered protein into the intermembrane space of the mitochondria. Antibodies raised against purified cytochrome *c* heme lyase or a synthetic antigen constructed on the basis of the predicted amino acid sequence, might be utilized to determine the subcellular location of the protein in the *cyt-2-1* mutant. From this it could be concluded whether the *cyt-2-1* mutation impairs transport of cytochrome *c* heme lyase into mitochondria.

It is likely that several functional domains exist in the cytochrome *c* heme lyase protein. Encoded by a nuclear gene, the protein presumably has a sequence that is responsible for the transport of the polypeptide into its proper location in mitochondria. At present, it is not known whether cytochrome *c* heme lyase is synthesized as a precursor with a cleavable presequence or if some internal sequence guides it into mitochondria. Unlike most of the proteins that are imported into mitochondria the amino terminus of the *cyt-2* gene product contains both acidic and basic residues instead of the usual basic residues (Douglas *et al.*, 1986). However, there are other exceptions to the general pattern, namely the yeast cytochrome *c* heme lyase (Dumont *et al.*, 1987), as well as the 17-kiloDalton subunit VI of the yeast ubiquinol-cytochrome *c* reductase (van Loon *et al.*, 1984). Thus, as noted previously, complete absence of acidic residues from the amino terminal portion of mitochondrial precursor proteins is not a prerequisite for import into the organelle (van Loon *et al.*, 1984).

Cytochrome *c* heme lyase protein appears to be bound to the mitochondrial membrane system with its catalytic domain exposed to the intermembrane space (Nicholson *et al.*, 1988). Thus, it is likely that the cytochrome *c* heme lyase protein has a membrane bound domain. No extended stretches of hydrophobic amino acids characteristic of the membrane spanning domains have been found in the *cyt-2* protein. The hydropathy plot of the *CYC3* gene product shows that yeast cytochrome *c* heme lyase is also predominantly hydrophilic. However, this does not exclude the possibility that the protein associates with the membrane via protein-protein interactions or via covalently linked nonprotein anchors such as lipid, fatty acids or a glycosyl-phosphatidylinositol (GPI) (Ferguson and Williams, 1988). Several lines of evidence suggest that in addition to its catalytic function cytochrome *c* heme lyase is the receptor for cytochrome *c* during its import into mitochondria (Nargang *et al.*, 1988; Nicholson *et al.*, 1988; Stuart, 1989). Nicholson *et al.* (1988) suggest that the membrane-embedded domain of the lyase may be responsible for binding apocytochrome *c*, while its intermembrane-space exposed domain catalyzes holocytochrome *c* formation. At present it is not known whether cytochrome *c* heme lyase is bound to the outer or inner mitochondrial membrane. It has been suggested that, if the enzyme is located in the vicinity of the outer membrane, it could bind apocytochrome *c* without prior translocation of the entire precursor across the outer membrane (Nicholson *et al.*, 1988).

Two short domains in the cytochrome *c* heme lyase sequence could be involved in binding heme (Fig. 18). The sequences are relatively well conserved between yeast and *N. crassa* and contain cysteine. A similar repeat sequence, ..CPVDH.., has been found in the HAP1 regulatory protein of yeast (Fig. 24) (Pfeifer *et al.*, 1989), which mediates the heme induction of several genes encoding mitochondrial proteins (see Introduction). Pfeifer *et al.*, (1989) suggest that these sequences may be involved in binding heme, which

Figure 24. Potential heme-responsive domains in the yeast HAP1 protein and cytochrome *c* heme lyase of yeast and of *N. crassa*.

Partial amino acid sequence of the yeast HAP1 regulatory protein (Pfeifer *et al.*, 1989) and the yeast (Dumont *et al.*, 1987) and *N. crassa* cytochrome *c* heme lyase (CCHL) are shown. The sequences are depicted to align the motif CP(V/I)DH which is repeated seven times in the HAP1 protein and twice in both the yeast and the *N. crassa* cytochrome *c* heme lyase (CCHL). The CP(V/I)DH sequence is indicated in bold letters.

HAP1

(280) K**CPINH**AQAPPSAAAAATR

K**CPVDH**SAFSSGMVAPKEETPLPR

R**CPVDH**TMFSSGMIPPREDTSSQK

R**CPVDH**TMYSAGMMPPKDETPSPFST

K**AMIDH**NKHTMNPPQS

K**CPVDH**ANYMKDYPSDMANSSSNPAS

R**CPIDH**SSMKNTAALPASTHNTIPH
(439)

yeast
CCHL

(25) G**CPVMH**ESSSSSPPSS

E**CPVMQ**GDNDRINPLNNM (58)

N. crassa
CCHL

(34) S**CP-MH**NKTMDALSAHKPVTPAPEP (61)

(70) K**CPVNH**GAKDTLAAAAAAVAPKQPQ (90)

would result in unmasking the DNA binding domain of the HAP1 protein. Similarly, binding of the heme group by cytochrome *c* heme lyase could induce a conformational change in the enzyme structure and activate its catalytic domain.

Although nothing is known about the regulation of cytochrome *c* heme lyase expression, sequences resembling the yeast UAS (upstream activator sequence) segments were found upstream of the *cyt-2* coding sequence (Table 8). Similar sequences were also noted in the 5' noncoding region of the yeast *CYC3* gene (Dumont *et al.*, 1987) and in the *N. crassa cyc-1 gene* (Bottorff, 1990). In yeast these sequences have been implicated in controlling transcription of *CYC1* (iso-1-cytochrome *c*; Guarente *et al.*, 1984), and *CTT1* (catalase T; Spevak *et al.*, 1986) in response to the levels of catabolite and heme. In fact, it has been shown by DNaseI protection and methylation interference studies that heme activated HAP1 regulatory protein binds to the UAS1 sequence of *CYC1*, protecting a region from -269 to -247 (Pfeifer *et al.*, 1987). HAP1 contact sites within UAS1 appear to occur with bases past the CTCTTT (-269 to -265) motif, which is the only common feature of these sequences (table 8). This is not too surprising though, since HAP1 also binds to another UAS sequence, located upstream of the *CYC7* gene for iso-2-cytochrome *c*, which does not exhibit any similarity to the UAS1 sequence. Although the precise positions of the contacts differ, the gross features of the interaction of HAP1 with the two sites are similar (at both sites HAP1 binds predominantly to one side of the DNA helix and extends over two full helical turns; contacts are made in both the major and minor grooves; single major groove contact is made on the opposite side of the helix at one end of each binding site) (Pfeifer *et al.*, 1987). The most highly conserved motif CTCTTT appears also in the *cyt-2* gene at positions -66,

-206, and -238 (Fig. 15). The 3' end of the -206 sequence overlaps by two base pairs with the 5' end of the cDNA sequence which may be equivalent with the beginning of the transcript (Fig. 15). The significance of these potential regulatory sequences in the *cyt-2* gene is unclear, especially in view of the fact that the 2.1 kb HindIII fragment (Fig. 13), which does not contain these sequences, is capable of rescuing the *cyt-2-1* phenotype. However, the latter observation does not exclude these sequences from playing a regulatory role since the possible transcription start site, deduced from the *cyt-2* cDNA sequence, is also not present on the HindIII fragment. It is conceivable that expression of the *cyt-2* gene in *Neurospora* transformants is regulated by sequences located near its integration sites, or that a low level of expression is achieved by other signals present on the fragment. Finally, the fast-growing *cyt-2-1* transformants might arise by homologous recombination, in which case all the regulatory elements for the expression of the *cyt-2* gene would be provided by its genomic counterpart. Both homologous and non-homologous integration of transforming DNA into chromosomes have been found in *Neurospora* transformants (Case, 1986; Paietta *et al.*, 1987). To determine which type of integration predominates in the *cyt-2-1* cells transformed with the 2.1 kb HindIII fragment, Southern analysis of their DNA would have to be performed.

Other regulatory sequences such as a "TATA" box (Grosschedl and Birnstiel, 1980) and a "CAAT" box (Efstradiatis *et al.*, 1980) were not found upstream from the putative transcription start site of the *cyt-2* gene. This is not surprising though, since most *Neurospora* genes that have been analyzed lack these elements. Also, no sequences resembling the eukaryotic polyadenylation recognition site AATAAA (Proudfoot and Brownlee, 1976) are found in *Neurospora* genes. However, a conserved element, TTTTCACTGC, found at the 3' end of several transcripts such as *con-10* (conidiation-specific gene; Roberts *et al.*, 1988), MT (copper metallothionein gene; Munger *et al.*, 1985), and *tub-2* (β-tubulin; Orbach *et al.*, 1986) could be a polyadenylation signal. A similar sequence, TTTTCAATGT, is present in the *cyt-2* gene 54 nucleotides upstream from the 3' end of the *cyt-2* transcript.

The finding that the *cyt-2* gene encodes cytochrome *c* heme lyase provides a direct explanation for the cytochrome *c* deficiency in the *cyt-2-1* mutant strain of *N. crassa*. As mentioned before, the enzyme catalyzes attachment of the heme group to apocytochrome *c*, which is a prerequisite for its transport into mitochondria (Hennig and Neupert, 1981; Nicholson *et al.*, 1987). In the *cyt-2-1* mutant apocytochrome *c* can not be converted to holocytochrome *c* and is rapidly degraded in the cytoplasm (Nargang *et al.*, 1988). However, the absence of cytochrome aa_3 in the *cyt-2-1* mitochondria must be due to an indirect effect(s) since cytochrome aa_3 does not have a covalently attached heme group (Tzagoloff, 1982). There are many observations in both yeast and *Neurospora* suggesting that a severe deficiency of cytochrome *c* results in an absence of cytochrome aa_3. Certain alleles of the *cyc3* mutant of yeast cause a deficiency of cytochrome aa_3, particularly under glucose repressing conditions (Reilly and Sherman, 1965; Sherman *et al.*, 1965). Also, strains of yeast that are severely deficient in both the iso-1 and iso-2 forms of cytochrome *c*, due to mutations in the *cyc1* and *cyc7* loci, respectively, are also deficient in cytochrome aa_3 (Downie *et al.*, 1977a, 1977b). Similarly, the cytochrome *c*-deficient *cyc-1-1* (Stuart *et al.*, 1987) and *cyc-1-12* (Bottorff, 1990) mutants of *N. crassa*, which directly affect the cytochrome *c* protein, are also deficient in cytochrome aa_3 when grown at 37°C (H. Bertrand, personal communication). These observations lead to the conclusion that a functional cytochrome *c* is required for efficient expression and/or assembly of cytochrome *c* oxidase. However, absence of cytochrome *c* can be compensated by other signals. As mentioned before (see Introduction) *cyt-2-1 cyb-1-1* double mutants of *N. crassa* which have both cytochrome *c* and cytochrome *b* production affected, are deficient in cytochrome *c* but do contain cytochrome aa_3 (Bertrand and Collins, 1978). Thus, the relationship between the expression of the cytochromes *c* and aa_3 must be a complex one, involving many genes and regulatory signals.

In conclusion, the results of the study of the *cyt-2-1* mutation in *N. crassa* showed that it does not affect a regulatory function but an enzyme, CCHL, that is directly involved in the synthesis of cytochrome *c* and its transport into the mitochondria. This finding, however, has some interesting implications with regards to the regulation of the assembly of the cytochrome aa_3, which is impaired in *cyt-2-1* cells. At present it is not known whether cytochrome aa_3 formation is regulated directly by cytochrome *c* or by other regulatory factors. Study of the assembly of the cytochrome *c* oxidase subunits may provide some insight into this complex process and explain cytochrome aa_3 deficiency seen in the *cyt-2-1* mutant.

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Appendix

LB Broth:

10 g bacto-tryptone (Difco)
5 g yeast extract (Difco)
5 g NaCl
2 g $MgSO_4.2H_2O$

The solution was made up to 1 l with water and sterilized by autoclaving for 20 minutes at 121°C. Plating medium was made by adding bacto-agar (Difco) to 1.5% prior to autoclaving. Top agarose was made by adding agarose to 0.7%.

Top Agar:

20 ml 50X Vogel's (Davis and de Serres, 1970)
182 g Sorbitol
1ml 1X trace elements (Davis and de Serres, 1970)
10 ml vitamins mix
15 g agar

Supplements were added as required before the solution was made up to 900 ml. After autoclaving, 100ml of 10X sugars solution was added. For determining spheroplast viability, no benomyl was added. For transformations, 2 ml of benomyl solution (0.25 mg/ml in 95% ethanol) was added at the same time as the 10X sugars solution.

SM Buffer:

5.8 g NaCl
2.0 g $MgSO_4 \cdot 2H_2O$
50 ml 1 M Tris-Cl, pH 7.5
5 ml 2% gelatin
Add dH_2O to 1 l and autoclave.

2X TY Medium:

10 g yeast extract (Difco)
16 g bacto-tryptone (Difco)
10 g NaCl
Add dH_20 to 1 l and autoclave.

Vitamins Mix:

30 mg riboflavin
100 mg thiamine
75 mg pyridoxine
5 ml *p*-aminobenzoic acid (2 mg/ml)
200 mg choline chloride
1 ml folic acid (5 mg/ml)
4 g myo-inositol
0.5 g adenine sulfate
0.5 g uracil
0.5 g L-methionine
0.5 g L-arginine
1 g L-lysine
0.1 g L-threonine
0.5 g L-serine
0.2 g L-homoserine
0.5 g L-histidine
0.1 g L-isoleucine
0.2 g L-valine
0.2 g L-leucine
0.5 g L-proline

The riboflavin was dissolved in 100 ml of water containing 4 drops of 5N NaOH before adding to the rest of the solution. After adding water to 1 l, the solution was filter sterilized and stored in dark bottles at 4°C.

10X Sugars Solution

200 g L-sorbose
5 g D-fructose
5 g D-glucose
2 g myo-inositol

This was made up to 1 l with water and sterilized by autoclaving.

Vogel's-Sorbose Plates

20 ml 50X Vogel's (Davis and de Serres, 1970)
1 ml Biotin Solution (12.5 mg/ml in 50% ethanol)
1 ml 1X trace elements (Davis and de Serres, 1970)
15 g agar

Supplements were added as required and the solution was made up to 900 ml with water. After autoclaving, 100 ml of 10X sugars solution was added prior to pouring the plates.

50X Denhardt's:

5 g ficoll
5 g polyvinylpyrrolidone
5 g BSA

The solution was stored at -20°C after adding water to 500 ml.

Oligolabeling Buffer:

Oligolabeling buffer was made by mixing solutions A:B:C in a ratio of 100:250:150 and stored at -20°C.

Solution A: 1 ml of solution O + 18 ml of β-mercaptoethanol + 5 ml each of 0.1 M solutions of dATP, dGTP and dTTP in 3 mM Tris-Cl, pH 7.0, 0.2 mM EDTA (stored at -20°C).

Solution B: 2 mM Hepes, pH 6.6 (titrated with 4 M NaOH and stored at 4°C).

Solution C: Hexadeoxyribonucleotides (P-L Biochemicals) made to 90 OD units/ml in 3 mM Tris-Cl, pH 7.0, 2 mM EDTA (stored at -20°C).

Solution O: 1.25 M Tris-Cl, pH 8.0, 0.125 M $MgCl_2$ (stored at 4°C).

Regular Sequencing Mixes:

A mix

40 µl 0.5 mM dCTP
40 µl 0.5 mM dGTP
40 µl 0.5 mM dTTP
40 µl 10X pol buffer
140 µl dH_2O
1 µl 10 mM ddATP

C mix

4 µl 0.5 mM dCTP
40 µl 0.5 mM dGTP
40 µl 0.5 mM dTTP
40 µl 10X pol buffer
120 µl dH_2O
2 µl 10 mM ddCTP

G mix

40 µl 0.5 mM dCTP
4 µl 0.5 mM dGTP
40 µl 0.5 mM dTTP
40 µl 10X pol buffer
110 µl dH_2O
4 µl 10 mM ddGTP

T mix

40 µl 0.5 mM dCTP
40 µl 0.5 mM dGTP
4 µl 0.5 mM dTTP
40 µl 10X pol buffer
100 µl dH_2O
8 µl 10 mM ddTTP

Chase:

20 µl 10 mM dATP
20 µl 10 mM dCTP
20 µl 10 mM dGTP
20 µl 10 mM dTTP
120 µl dH_2O

10X Pol Buffer:

0.7 ml 1 M Tris-Cl, pH 8.0
142 mg $MgCl_2 \cdot 6H_2O$
1 ml 5 M NaCl
Add dH_2O to 10 ml.

Dye-Formamide Mix:

10 mg Xylene Cyanole FF (Kodak)
10 mg bromphenol blue
400 ml 0.25 M EDTA, pH 8.0
9.5 ml deionized formamide

6% polyacylamide sequencing gels:

6 ml 40% (w/v) acrylamide/bisacrylamide (19/1)
20 g urea
4 ml 1 M Tris-borate, pH 8.3, 20 mM EDTA
15 ml dH_2O

The urea was dissolved by placing the solution in a 50°C water-bath and then filtered prior to use.